UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS
Departamento de Bioquímica y Biología Molecular

TESIS DOCTORAL

Efecto del procesado térmico, de presión y enzimático sobre alérgenos de frutos secos y su detección por PCR en tiempo real

Effect of thermal, pressure and enzymatic processing on tree nut allergens and their detection by real-time PCR

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

África Sanchiz Giraldo

Directores

Carmen Cuadrado Hoyo
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Memoria presentada por África Sanchiz Giraldo para optar al Título de Doctor por la Universidad Complutense de Madrid

Directoras de la Tesis: Dra. Carmen Cuadrado Hoyo, Departamento de Tecnología de Alimentos, INIA y Dra. Rosario Linacero de la Fuente, Departamento de Genética, Fisiología y Microbiología, Facultad de CC Biológicas, UCM

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CERTIFICAN:

Que la tesis titulada “Efecto del procesado térmico, de presión y enzimático sobre alérgenos de frutos secos y su detección por PCR en tiempo real” de la que es autora África Sanchiz Giraldo, ha sido realizada bajo su dirección en el departamento de Tecnología de Alimentos del INIA y en el departamento de Genética, Fisiología y Microbiología de la Facultad de CC Biológicas (UCM), y cumple las condiciones exigidas para optar al grado de Doctor por la Universidad Complutense de Madrid.

Madrid, septiembre de 2018

Fdo. Carmen Cuadrado

Fdo. Rosario Linacero

FINANCIACIÓN PARA EL DESARROLLO DE LA PRESENTE TESIS DOCTORAL

La presente Tesis Doctoral se ha realizado en el marco del proyecto AGL2012- 39863-C02 del Ministerio de Economía y Competitividad (MINECO).

Durante la realización de la presente Tesis, su autora fue beneficiaria de una ayuda para contratos predoctorales para la formación de doctores del Ministerio de Economía y Competitividad (MINECO) en la convocatoria de 2013, referencia BES-2013-065833.

Además, su autora ha disfrutado de dos ayudas para estancias científicas, ambas en el laboratorio de la Dra. Natalija Novak, en el Departamento de Dermatología y Alergia de la Universidad de Bonn, Medical Center (UKB, Bonn, Alemania). La primera de ellas, realizada durante el períodos marzo-junio de 2016 fue financiada por el Ministerio de Economía y Competitividad (MINECO), en la convocatoria de "Ayudas a la movilidad predoctoral para la realización de estancias breves en centros de I+D 2015". La segunda estancia fue realizada en el período abril-octubre de 2017 y financiada con una ayuda *Medium Term Research Fellowship* 2016, otorgada por la Academia Europea de Alergia e Inmunología Clínica (EEACI).

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A mi familia
A Santos

"Te salgo a buscar, quimera. Mariposa de papel…
Te pienso seguir buscando, la vida entera."

J. Drexler

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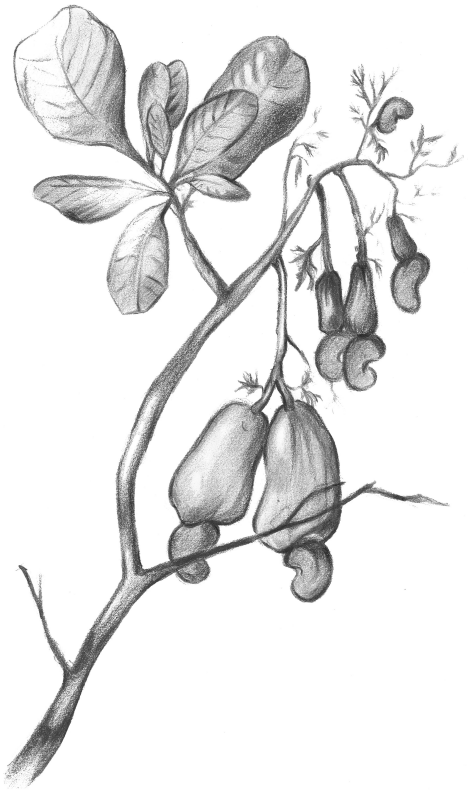
RESUMEN



Los frutos secos son uno de los alimentos más frecuentemente implicados en reacciones alérgicas. El procesado térmico y no térmico de los alimentos puede producir cambios estructurales en las proteínas alergénicas modulando su capacidad antigénica. En esta Tesis Doctoral, titulada "*Efecto del procesado térmico, de presión y enzimático sobre alérgenos de frutos secos y su detección por PCR en tiempo real"*, se ha analizado el impacto del procesado sobre la inmunoreactividad de pistacho y anacardo. Los tratamientos utilizados han sido térmicos, de cocción (30 y 60 min) o con aplicación de presión (autoclave a 121^{o}C/1.18 atm o 138^{o}C/2.56 atm durante 15 y 30 min), así como la combinación de éstos con hidrólisis enzimática con proteasas y sonicación. Se han empleado técnicas de inmunodetección *in vitro*, LC-MS/MS, pruebas cutáneas en pacientes alérgicos y ensayos de liberación de mediadores de la respuesta alérgica.

La cocción no reduce significativamente la capacidad de unir IgE de pistacho y anacardo con respecto al control sin tratar , aunque sí disminuye la capacidad de inducir degranulación de basófilos. Al aplicar calor y presión, especialmente a 138^{o}C/2.56 atm 30 minutos, la capacidad de anacardo y pistacho de unir IgE tanto *in vitro* como en la superficie de células efectoras disminuye considerablemente. La digestión enzimática desencadena la proteólisis de alérgenos de anacardo y en especial de pistacho, reduciendo su reactividad frente a anticuerpos IgE. La combinación de tratamientos térmicos con hidrólisis enzimática representa una eficiente estrategia para reducir la inmunoreactividad de estos frutos. Se han identificado péptidos de algunos alérgenos de anacardo y pistacho que resisten incluso al tratamiento combinado de calor, presión y digestión enzimática.

Se ha estudiado la influencia de los tratamientos de cocción y autoclave sobre compuestos fenólicos, actividad antioxidante y propiedades funcionales de pistacho, anacardo y castaña. El contenido de compuestos fenólicos se mantiene en los frutos secos procesados, e incluso se incrementa en castaña autoclavada a 138^{o}C 30 min. La actividad antioxidante tampoco resulta afectada en los tres frutos secos tratados. Se ha observado que tras la aplicación del procesado térmico y de presión, se mantienen algunas de las propiedades tecno-funcionales de las harinas de pistacho, anacardo y castaña, lo que permitiría su uso en la industria alimentaria.

La normativa de seguridad alimentaria relacionada con riesgo alergénico exige identificar y etiquetar correctamente la presencia de frutos secos en alimentos. Por ello, es esencial desarrollar un método de detección sensible, específico, preciso y reproducible. En esta Tesis Doctoral se han desarrollado protocolos, basados en la detección de ADN por PCR en tiempo real, que permiten la detección específica de nuez, pistacho y anacardo, usando como

secuencias diana genes que codifican alérgenos. Se han empleado distintas químicas de reacción: SYBR Green y sondas de hidrólisis LNA y TaqMan. Se han desarrollado métodos de detección de nuez (Jug r 3) con LOD de 100 ppm empleando SYBR Green, de pistacho (Pis v 1) con LOD de 10 ppm con sondas LNA y de anacardo (Ana o 1) con LOD de 10 ppm con ambos tipos de sondas, TaqMan y LNA. Se ha evaluado el efecto del procesado térmico y de presión sobre la detección de estos frutos secos. La cocción y las altas presiones hidrostáticas (HHP) no afectan a la detección. Sin embargo, la aplicación de temperatura y presión afecta a la integridad del ADN, dificultando la detección de las dianas. A pesar de ello, se ha conseguido detectar y cuantificar por PCR en tiempo real, la presencia de nuez, pistacho y anacardo en muestras procesadas. Estos métodos se han validado en alimentos comerciales procesados obteniendo resultados más precisos que con ELISA.

En conclusión, el tratamiento de autoclave a 138^{o}C durante 30 minutos reduce, hasta prácticamente anular, la capacidad inmunoreactiva de pistacho y anacardo. Algunos péptidos de alérgenos principales de estos frutos secos resisten a este tratamiento incluso en combinación con hidrólisis mediante proteasas. Además, las harinas tratadas de anacardo, pistacho y castaña, con probada inmunoreactividad reducida, mantienen el contenido en compuestos fenólicos y su actividad antioxidante. Se han desarrollado métodos de detección por PCR en tiempo real que permiten detectar, sensible y específicamente, trazas de nuez, pistacho y anacardo, incluso tras ser sometidos a tratamiento térmico y de presión. Se ha demostrado la aplicación de los métodos analizando alimentos comerciales procesados.

SUMMARY

Tree nuts are one of the ingredients most frequently involved in allergic reactions. Thermal and non-thermal food processing can produce structural changes in allergenic proteins, modifying their antigenic capacity. In this Doctoral Thesis, entitled "Effect of thermal, pressure and enzymatic processing on tree nut allergens and their detection by real-time PCR", the impact of processing on pistachio and cashew immunoreactivity has been analysed. Thermal treatments were used, boiling (30 and 60 min) or heat and pressure (autoclave at 121^{o}C / 1.18 atm or 138^{o}C / 2.56 atm for 15 and 30 min), as well as the combination of these with enzymatic hydrolysis using proteases under sonication. *In vitro* immunodetection techniques, LC-MS / MS, skin prick tests in allergic patients and release assays of allergic response mediators have been used.

Boiling does not significantly reduce the IgE-binding capacity of pistachio and cashew compared to untreated control although it decreases the ability to induce degranulation of basophils. When heat and pressure is applied, especially at 138^{o}C / 2.56 atm 30 minutes, the ability of cashew and pistachio to bind IgE, both *in vitro* and on the surface of effector cells, decreases considerably. The enzymatic digestion triggers the proteolysis of cashew allergens and especially of pistachio, reducing its reactivity against IgE antibodies. The combination of thermal treatments with enzymatic hydrolysis represents an efficient strategy to reduce the immunoreactivity of these tree nuts. Some peptides from cashew and pistachio allergenic proteins have been identified as resistant even after the combined treatment of heat, pressure and enzymatic digestion.

The influence of boiling and autoclaving on phenolic compounds, antioxidant activity and functional properties of pistachio, cashew and chestnut has also been studied. The content of phenolic compounds is maintained in processed nuts and it is even increased in chestnut autoclaved at 138^{o}C 30 min. The antioxidant activity is not affected in the three processed nuts. It has been observed that after the application of thermal and pressure processing, some of the techno-functional properties of the pistachio, cashew and chestnut flours are maintained, which allows their use in the food industry.

Food safety regulations concerning allergen risk require the identification and proper labelling of nuts when they are present in commercial foods. Therefore, it is essential to develop a sensitive, specific, accurate and reproducible detection method. In this Doctoral Thesis, protocols based on the detection of DNA by real-time PCR have been developed, which allow the specific detection of walnut, pistachio and cashew nuts, using allergen coding genes as target sequences. Different reaction chemistries have been used: SYBR Green and LNA and TaqMan hydrolysis probes. Methods for the detection of walnut (Jug r 3) with LOD of 100 ppm using SYBR

Green, for the detection of pistachio (Pis v 1) with LOD of 10 ppm using LNA probes and for the detection of cashew (Ana o 1) with LOD of 10 using both LNA and TaqMan probes, have been developed. The effect of thermal processing and pressure on the detection of these target sequences of nuts has been also evaluated. Boiling and high hydrostatic pressures (HHP) did not affect detection. However, the application of temperature affects the DNA integrity, making more difficult to detect these target sequences. In spite of this, it has been possible to detect and quantify, by real-time PCR, the presence of walnut, pistachio and cashew nuts in processed samples. These methods have been validated in processed commercial foods obtaining more accurate results than with ELISA.

In conclusion, autoclaving at 138^{o}C for 30 minutes reduces, until practically abolish, the immunoreactive capacity of pistachio and cashew nuts. Some peptides of the main allergens of these nuts resist this treatment even in combination with protease hydrolysis. In addition, the treated cashew, pistachio and chestnut flours, with proven reduced immunoreactivity, maintain the content of phenolic compounds and their antioxidant activity. Real-time PCR detection methods have been developed to detect, sensitively and specifically, traces of walnut, pistachio and cashew, even after being subjected to heat and pressure treatment. Applicability of the methods has been successfully validated by analysing processed commercial foods.

INTRODUCCIÓN

1. ALERGIA ALIMENTARIA

La Academia Europea de Alergia e Inmunología Clínica (EAACI, siglas de *European Academy of Allergy and Clinical Inmunology),* clasifica las reacciones adversas a los alimentos, producidas tras la ingestión, contacto o inhalación de un determinado alimento, como tóxicas y no tóxicas (Figura 1). Las reacciones tóxicas se producen por la presencia de toxinas y contaminantes microbianos o farmacológicos que pueden afectar a cualquier individuo siempre que la cantidad ingerida sea suficiente. Las reacciones adversas no tóxicas sólo se dan en individuos susceptibles y pueden dividirse en mediadas o no mediadas por el sistema inmune (hipersensibilidad o intolerancia alimentaria respectivamente).

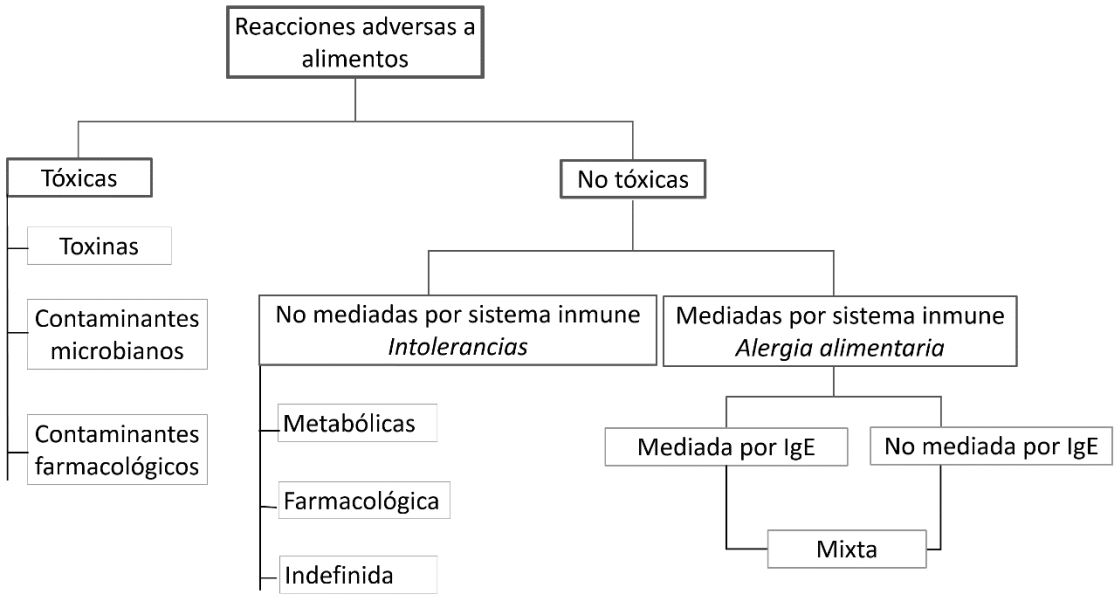


Figura 1. Clasificación de reacciones adversas a alimentos (EAACI).

La alergia alimentaria puede definirse como una reacción adversa o hipersensibilidad clínica reproducible a un alimento (alérgeno alimentario) mediada por el sistema inmunitario, que a su vez pueden involucrar o no la producción de anticuerpos inmunoglobulina E (IgE) (Yu *et al*, 2016). Se cree que en las alergias no mediadas por IgE participan las células T y los eosinófilos (alergia mediada por células). Producen trastornos digestivos inflamatorios agudos o crónicos y gastroenteropatías inducidas por proteínas de la dieta, y no desencadenan degranulación de mastocitos y basófilos (Cianferoni & Spergel, 2009).

Las alergias alimentarias mediadas por anticuerpos IgE específicos frente un determinado alérgeno tiene mayor repercusión clínica porque son, en muchos casos, responsables de reacciones adversas muy severas e incluso letales (hipersensibilidad de tipo I)

(Burks *et al*, 2008). Los síntomas pueden ser dermatológicos (urticaria, angioedema o dermatitis/eczemas atópicos), gastrointestinales (diarrea, dolor abdominal, cólicos, vómito, estreñimiento), respiratorios (rinorrea, tos, disnea, estornudos) o circulatorios (colapso cardiovascular, hipotensión) (Muraro *et al*, 2014b; Sicherer *et al*, 2017). Se ha documentado que los pacientes alérgicos sufren efectos psicológicos y emocionales, además de físicos y sociales (Cummings *et al*, 2010). La alergia a alimentos es la principal causa de urgencia hospitalaria, debida a anafilaxis, en países occidentales (Bock *et al*, 2007; Keet & Wood, 2007; Tejedor-Alonso *et al*, 2015; Grabenhenrich *et al*, 2016). La mayoría de casos de alergia alimentaria se asocian a un limitado número de fuentes alergénicas. En Europa, se ha establecido una lista de 14 alimentos asociados más frecuentemente a casos de alergia: leche de vaca, huevo, frutos de cáscara, soja, cacahuete, cereales con gluten, pescado, moluscos, crustáceos, apio, mostaza, sésamo, lupino y dióxido de azufre/sulfitos (reglamento (EU) nº 1169/2011 del Parlamento Europeo y del Consejo del 25 de octubre de 2011) (Muraro *et al*, 2014a).

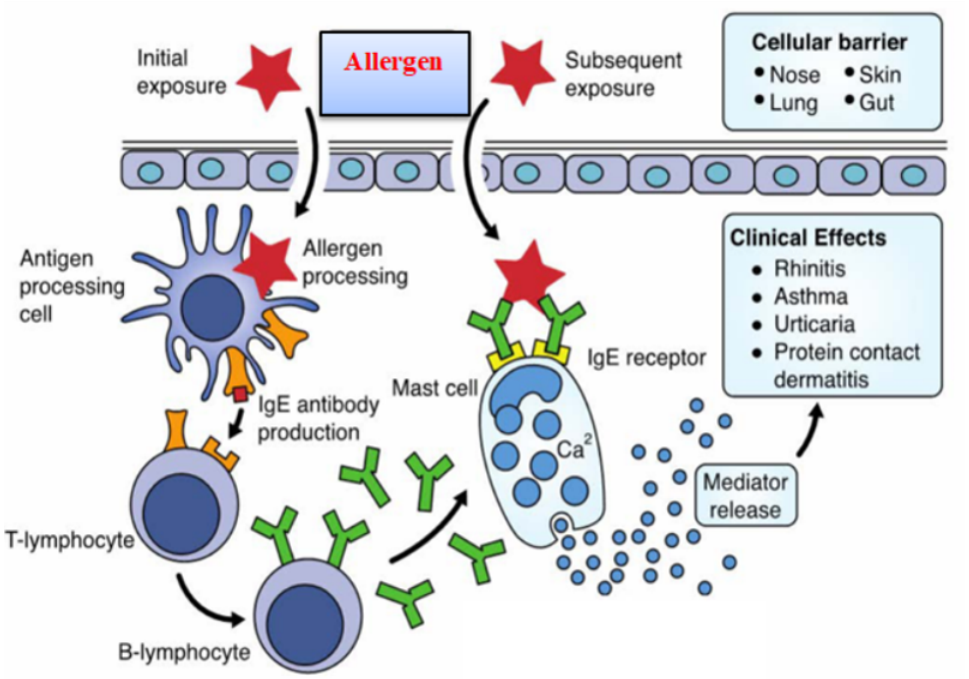


Figura 2. Representación esquemática del mecanismo de alergia alimentaria mediada por producción de IgE. Figura tomada de Liu, Navarro & Lopata (2016).

La aparición de síntomas derivados de la alergia alimentaria se precede de una fase asintomática de exposición al alérgeno o sensibilización, que puede darse en el tracto gastrointestinal, cavidad oral, piel o tracto respiratorio (Sampson *et al*, 2018). Tras la ingestión, el ácido estomacal y las enzimas digestivas degradan las proteínas alimentarias y los fragmentos o péptidos, inmunológicamente activos, salen del lumen a través de células epiteliales o células dendríticas y macrófagos, en las que son internalizados y procesados. Estas células unirán los

fragmentos peptídicos a unos receptores denominados MHC de clase II. La célula presentadora interaccionará con las células T *naive* colaboradoras (Th) a través de ese antígeno en MHC II y serán activadas, promoviendo la liberación de moléculas co-estimuladoras, del tipo citoquinas, que provocarán la producción de anticuerpos IgE específicos por parte de las células B y promoverán el reclutamiento y activación de basófilos, mastocitos y eosinófilos. El fragmento Fc de estos anticuerpos IgE se une a la subunidad α de alta afinidad de los receptores FcεRI expresados en las membranas de mastocitos, basófilos y otras células (Figura 3) (MacGlashan, 2005). En la segunda fase de re-exposición al antígeno o de provocación, el alérgeno es reconocido y unido específicamente por la IgE adherida al receptor FcεRI y se desencadena la liberación de mediadores pro-inflamatorios (como histamina), responsables de la sintomatología alérgica en el individuo (Sicherer, 2002; Sicherer & Sampson, 2018).

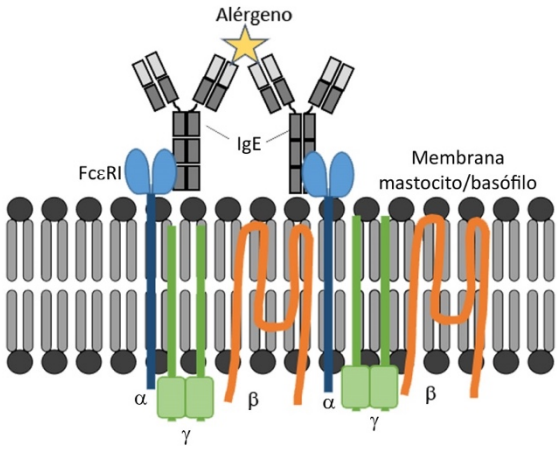


Figura 3. Esquema de dos receptores FcεRI en la membrana celular y anticuerpos IgE unidos a las subunidades α.

Para llevar a cabo una evaluación de la alergenicidad de los alimentos, es decir, de la capacidad de los mismos para unir IgE de sueros de pacientes, es necesario una correcta caracterización clínica del grupo de estudio. Las pruebas de provocación oral controlada (OFC, del inglés *Oral Food Challenge*) son esenciales para verificar la reactividad alergénica frente a un ingrediente, siempre que no haya historia de anafilaxia. Pueden ser abiertas, cuando el paciente y el personal sanitario identifican previamente el alimento; simple ciego controlado con placebo, cuando el paciente no identifica el alimento/placebo; y el doble ciego controlado por placebo (DBPCFC), considerado la prueba más objetiva (*"gold standard")*, en el que ni el personal sanitario ni el paciente identifican previamente el alimento ni el placebo (Sicherer & Sampson, 2018). Complementariamente, uno de los métodos más empleados en clínica para determinar niveles de IgE total y específico de alérgeno es el inmunoanálisis enzimático con marcaje fluorimétrico (FEIA) (Poms *et al*, 2004).

Se estima que más del 2% pero menos del 10% de la población general sufre alergia alimentaria, siendo más elevada en población infantil que adulta (Muraro & Roberts, 2014; Sicherer *et al,* 2017). La prevalencia de la alergia a alimentos se ha incrementado en las últimas décadas en los países desarrollados, efecto atribuido a diversas causas ambientales y nutricionales tales como hábitos de alimentación, higiene y otros factores como el sexo o genéticos (Sicherer & Sampson, 2018; Leung *et al*, 2018; Ojeda *et al*, 2018). La prevalencia de la alergia a cada uno de los alimentos varía según la región geográfica, la población o la edad de los pacientes (Nwaru *et al*, 2014). Las diferencias entre las metodologías empleadas en los estudios epidemiológicos dificultan el establecimiento de la misma. De manera general, la prevalencia obtenida cuando se evalúa únicamente la sintomatología que describen los pacientes es considerablemente superior a la obtenida mediante la historia clínica y las pruebas de provocación con el ingrediente alergénico (Rona *et al*, 2007; Nwaru *et al*, 2014).

La eliminación total del ingrediente alergénico de la dieta del paciente es la única medida preventiva para evitar los síntomas de la alergia. En el caso de un contacto accidental por la presencia de alérgenos ocultos, la administración de adrenalina o epinefrina inyectable será necesaria en aquellos individuos que sufran reacciones más graves, mientras que los antihistamínicos, glucocorticoides o antileucotrienos serán aplicados para paliar los síntomas más leves (Yu *et al*, 2016). En los últimos años, se ha intensificado la investigación en inducción de tolerancia o inmunoterapia específica de alérgeno (oral, sublingual, subcutánea, epicutánea), cuyo objetivo es inducir en los individuos desensibilización a alimentos específicos. Se requiere más investigación para llegar a comprender la complejidad del fenómeno de tolerancia de antígenos alimentarios, ya que se ha documentado la posible aparición de reacciones graves (Jutel *et al,* 2015; Sampson *et al,* 2018; O'Konek *et al,* 2018; Ojeda *et al,* 2018).

1.1. Proteínas alergénicas de origen vegetal

Los alérgenos alimentarios son los antígenos que desencadenan la alergia; principalmente proteínas o glicoproteínas, aunque no de forma exclusiva, que reaccionan con inmunoglobulinas IgE de la superficie de mastocitos y basófilos. Los alérgenos se unen a estos anticuerpos a través de una región concreta denominada epítopo (Sicherer, 2002; Smeekens *et al*, 2018). Los epítopos reconocidos por los anticuerpos pueden ser conformacionales, aquellos que requieren una estructura peptídica tridimensional que a menudo implica segmentos aminoacídicos discontinuos en la estructura proteica, o epítopos secuenciales/lineales, generalmente péptidos cortos de unos 8-10 aminoácidos contiguos (Liu & Sathe, 2018).

Los panalérgenos son proteínas alergénicas distribuidas ampliamente en la naturaleza, que comparten regiones aminoacídicas, estructuras y funciones biológicas muy conservadas. Frecuentemente, los epítopos de los alérgenos se encuentran en regiones importantes para su función, lo que propicia encontrar secuencias homólogas en alérgenos en distintas especies, y explica la sensibilización en los pacientes para alimentos de especies tanto cercanas como taxonómicamente no relacionadas (Hauser *et al*, 2010). Este fenómeno inmunológico se denomina reactividad cruzada, en el que los anticuerpos IgE específicos de un alérgeno determinado reconocen una proteína en otra especie con estructura aminoacídica y/o tridimensional homóloga (Bonds *et al*, 2008).

Desde un punto de vista inmunológico, los alérgenos pueden clasificarse de acuerdo a la vía de sensibilización. Los alérgenos de clase I son aquellos cuya sensibilización se produce a través del tracto gastrointestinal tras la ingestión de un alimento. Se caracterizan por ser altamente estables a la temperatura, al tratamiento enzimático (proteasas) y al ácido. Los de clase II producen reacciones alérgicas en individuos previamente sensibilizados a alérgenos inhalados, que presentan reactividad cruzada con el alérgeno alimentario. Suelen ser alérgenos más sensibles a la temperatura y la digestión enzimática, y se encuentran frecuentemente asociados a polinosis (Harrer *et al*, 2010; Han *et al*, 2012; Geiselhart *et al*, 2018). La clasificación de alérgenos de origen vegetal más empleada se realiza en base a homologías en la estructura y la función de las proteínas, obteniéndose unas pocas familias y superfamilias fundamentalmente de proteínas de reserva de la semilla y de defensa de la planta (Breiteneder & Radauer, 2004).

1.1.1. Superfamilias cupina y prolamina

Las leguminas (globulinas 11S), vicilinas (globulinas 7S) y germinas (globulinas <7S) son proteínas de reserva que pertenecen a la superfamilia de las cupinas (Mills *et al*, 2004; Barre *et al*, 2018). Se caracterizan por una estructura en barril β y sufren procesado proteolítico dando lugar a una gran variedad de isoformas. Las leguminas son proteínas hexaméricas cuyo precursor se procesa formando trímeros en el retículo endoplasmático y posteriormente se escinden en una subunidad ácida, de 30-40 kDa, que se une a otra básica de unos 20 kDa mediante un puente disulfuro. Las vicilinas son moléculas triméricas compuestas de subunidades de entre 40 y 80 kDa.

La superfamilia de las prolaminas incluye las albúminas 2S, proteínas de reserva de pequeño peso molecular y ricos en estructura tipo α-hélice. Comparten de 6 a 8 residuos de cisteínas que conforman los puentes disulfuro, dando lugar a una estructura compacta (Crespo

et al, 2006; Moreno & Clemente, 2008). Dentro de esta superfamilia se incluyen también las proteínas transportadoras de lípidos no específicas (nsLTP), presentes en muchas frutas que mediante la presencia de 4 puentes disulfuro conservados, forman una cavidad interior en la que albergan ácidos grasos, fosfolípidos, etc (Geiselhart *et al*, 2018). Las α–amilasas y los inhibidores de tripsina son alérgenos de esta superfamilia, así como las prolaminas de cereales gliadinas y gluteninas (Breiteneder & Radauer, 2004; Hauser *et al,* 2010).

1.1.2. Proteínas del sistema de defensa de la planta

Las proteínas relacionadas con la patogénesis (PR) son proteínas que se inducen como respuesta ante estrés biótico y abiótico en la planta. No se trata de una superfamilia sino que incluye un grupo heterogéneo de proteínas alergénicas no relacionadas, que se encuentran en frutos secos, legumbres, cereales, frutas y otros vegetales (Breiteneder & Radauer, 2004). Son proteínas generalmente de bajo peso molecular, estables a bajo pH y resistentes a la proteólisis. Las quitinasas de clase I pertenecen a la familia PR-3 (alérgenos descritos en plátano, castaña o aguacate) y presentan reactividad cruzada con el alérgeno heveína de latex (Breiteneder & Radauer, 2004). Las proteínas tipo taumatina (TLP) y ciertas peroxidasas son también proteínas alergénicas del sistema defensivo de la planta (familias PR-5 y PR-9, respectivamente). Las proteínas PR-10 son importantes por mostrar reactividad cruzada con el alérgeno Bet v 1 de abedul, responsables de la frecuente alergia a ciertos alimentos vegetales en individuos alérgicos al polen (Breiteneder & Radauer, 2004; Hauser *et al,* 2010).

1.1.3. Familia de las profilinas

La familia de las profilinas incluye alérgenos de bajo peso molecular que se encuentran en todas las células eucariotas y cumplen un papel importante en movilidad celular (Breiteneder & Radauer, 2004). Son proteínas sensibles al calor y a la digestión gástrica, por lo que los síntomas suelen confinarse al síndrome alérgico oral (SAO). Las secuencias aminoacidicas de las profilinas están altamente conservadas en las plantas, con estructuras proteicas tridimensionales muy similares entre ellas. Los anticuerpos IgE frente a profilinas específicas de polen son capaces de unir con frecuencia las de alimentos (panalérgenos) (Wensing *et al*, 2002; Hauser *et al,* 2010).

1.2. Alergia a frutos secos

Los frutos secos pertenecen al grupo de alimentos responsables del 90% de las alergias alimentarias (Vanga & Raghavan, 2016). En España son uno de los alimentos más comúnmente

relacionados con alergia, solo por detrás de las frutas (Crespo *et al*, 2006; Fernandez Rivas, 2009). Los frutos secos más habitualmente consumidos pertenecen a las familias botánicas *Rosaceae* (almendra), *Anacardiaceae* (anacardo y pistacho), *Juglandaceae* (las nueces, las nueces de nogal negro y la nuez pecana), *Fagaceae* (castaña), *Pinaceae* (piñón), *Betulaceae* (avellana) y *Lecythidaceae* (nuez de Brasil) (Ros, 2010). Los cacahuetes también forman parte de este grupo, pertenecientes a la familia *Leguminoseae*, por el similar perfil nutricional que presentan (Ros, 2010). La alergia a estos alimentos es más frecuente en niños, perdura en la edad adulta y deriva con frecuencia a casos de anafilaxia (Couch *et al*, 2017; Weinberger & Sicherer, 2018; Geiselhart *et al*, 2018). En un meta-análisis europeo con información recopilada entre el 2000 y el 2012, se determinó que entre un 0.5% y un 1.3% de la población general sufría alergia a frutos secos, en función de la metodología de estudio llevada a cabo (Nwaru *et al,* 2014). Debido al elevado número de casos en los que se observa sensibilización cruzada a varios alérgenos de frutos secos, el tratamiento de eliminación del ingrediente en la dieta se extiende a todos los frutos secos una vez ésta ha sido diagnosticada (Smeekens *et al*, 2018).

En la tabla 1 se incluyen los alérgenos alimentarios descritos en frutos secos, según el WHO/IUIS, comité oficial de nomenclatura sistemática de alérgenos (www.allergen.org).

Tabla 1. Proteínas alergénicas alimentarias descritas en frutos secos.

Especie	Alérgeno	Naturaleza	PM (kDa)
Anacardo	Ana o 1	Vicilina 7S	50
Anacardium occidentale	Ana o 2	Legumina 11S	53
	Ana o 3	Albúmina 2S	14
Almendra	Pru du 3	nsLTP	9
Prunus dulcis	Pru du 4	Profilina	14
	Pru du 5	60sRP	10
	Pru du 6	Legumina 11S	63
Avellana	Cor a 1	PR-10	17
Corylus avellana	Cor a 2	Profilina	14
	Cor a 8	nsLTP	9
	Cor a 9	Legumina 11S	40
	Cor a 11	Vcilina 7S	48
	Cor a 12	Oleosina	17
	Cor a 13	Oleosina	15
	Cor a 14	Albúmina 2S	10
Cacahuete	Ara h 1	Vicilina 7S	64
Arachis hypogaea	Ara h 2	Albúmina 2S	17
	Ara h 3	Globulina 11S	60
	Ara h 5	Profilina	15
	Ara h 6 y 7	Albúmina 2S	15
	Ara h 8	PR-10	17
	Ara h 9	nsLTP	9.8
	Ara h 10 y 11	Oleosina	16 y 14
	Ara h 12 y 13	Defensina	12 y 11
	Ara h 14 y 15	Oleosina	17.5 y 17
	Ara h 16 y 17	nsLTP	8.5 y 11
Castaña	Cas s 5	Quitinasa	33
Castanea sativa	Cas s 8	nsLTP	10
	Cas s 9	HSP	17
Nuez	Jug r 1	Albúmina 2S	15
Juglans regia	Jug r 2	Vicilina	44
	Jug r 3	nsLTP	9
	Jug r 4	Legumina 11S	58
	Jug r 5	PR-10	20
	Jug r 6	Vicilina-like	47
	Jug r 7	Profilina	13
Nuez de Brasil	Ber e 1	Albúmina 2S	9
Bertholletia excelsa	Ber e 2	Globulina 11S	29
Nuez de nogal negro	Jug n 1	Albúmina 2S	18
Juglans nigra	Jug n 2	Vicilina 7S	55
	Jug n 4	Legumina 11S	58
Nuez pecana	Car i 1	Albúmina 2S	16
Carya illinoinensis	Car i 2	Vicilina 7S	55
	Car i 4	Legumina 11S	55
Piñón *Pinus pinea*	Pin p 1	Albúmina 2S	15
Pistacho	Pis v 1	Albúmina 2S	7
Pistacia vera	Pis v 2	Legumina 11S	50
	Pis v 3	Vicilina 7S	55
	Pis v 4	Fe/MnSOD	25
	Pis v 5	Legumina 11S	50

TLP: thaumatin like-protein, LTP: lipid transfer protein, HSP: heat shock protein, SOD: superoxide dismutase, PR: pathogenesis related; 60sRP: ribosomal protein 60S

1.2.1. Anacardo y pistacho

El anacardo (*Anacardium occidentale* L.) y el pistacho (*Pistacia vera* L.) pertenecen a la familia botánica Anacardiaceae. *A. occidentale* es un árbol de hoja perenne, nativo de Brasil, que presenta un característico pseudofruto (un pedúnculo) en cuyo final se encuentra una "nuez" con la semilla comestible, el anacardo (Figura 4A). La cascarilla del anacardo contiene una sustancia oleosa (CNSL, C*ashew Nut Shell Liquid*) con aplicaciones industriales, así como un aceite muy irritante, el urushiol (Hamilton & Zug, 1998; Akinhanmi & Atasie, 2008). En la actualidad, se producen unos 4.9 millones de toneladas de anacardo en todo el mundo, según la FAO (FAOSTAT 2018). El consumo de anacardo está asociado a una serie de beneficios para la salud, al ser rico en ácidos grasos insaturados, fenoles, fitoesteroles, tocoferoles y otros nutrientes (Mendes *et al*, 2016; Mattison *et al*, 2018). El ácido anacárdico es un compuesto fenólico del anacardo, asociado a ciertas actividades farmacológicas, como antimicrobiana, antiinflamatoria, anticancerígena e inhibidora de la acetilcolintransferasa (Suo *et al*, 2012; Mattison *et al*, 2018).

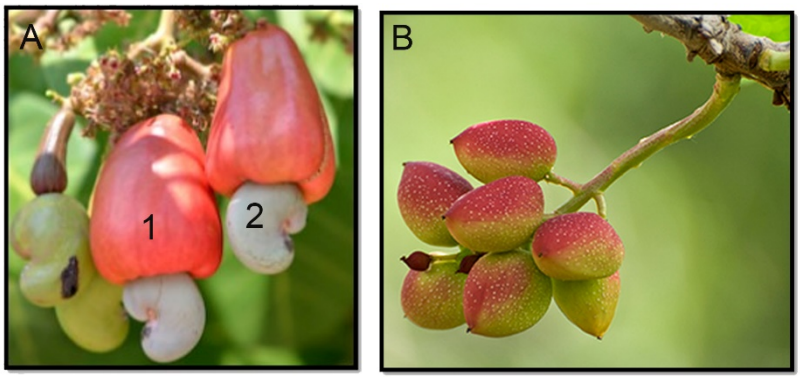


Figura 4. A) Frutos de anacardo. 1, pseudofruto; 2, nuez/semilla. B) Frutos de pistacho.

Las reacciones adversas que sufren los individuos sensibilizados a anacardo cubren síntomas que van desde cutáneos, respiratorios y gastrointestinales, hasta reacciones anafilácticas graves o letales (Rancé *et al*, 2003). Grabenhenrich y colaboradores (2016) realizaron un estudio en 10 países europeos, demostrando la relevancia del anacardo como causante de reacciones anafilácticas graves en niños y adolescentes. Otros autores han observado que la alergia a anacardo se ha incrementado en los últimos años, alcanzando entre el 5-6% de la prevalencia total de alergia a frutos secos en países europeos y provocando en algunos casos reacciones más severas que el cacahuete en niños alérgicos (Clark *et al*, 2007; Vetander *et al*, 2012; Johnson *et al*, 2014). En EEUU se ha documentado una incidencia de la alergia a anacardo del 30% en la población de pacientes testada, la misma que a nuez (Fleischer *et al*, 2005).

El pistacho, *Pistacia vera,* procede del pistachero, un árbol dioico nativo de Asia, que crece en áreas cálidas y áridas, soporta muy bien el frio y resiste la sequía, la salinidad y los suelos calizos. Se producen aproximadamente 1.1 millones de toneladas de pistacho en todo el mundo (FAOSTAT, 2018). En España el pistachero se ha adaptado con éxito fundamentalmente a secano o riego deficitario, alcanzando unas 12 000 hectáreas de cultivo (MAPAMA, 2017). La única parte comestible es una semilla tipo drupa con mesocarpo, agrupada en racimos (Figura 4B), que se separa fácilmente de la cáscara o endocarpio en la madurez y que se seca de forma natural en las zonas cálidas o aplicando calor forzado en las húmedas. El consumo de pistacho se ha asociado a un descenso en el riesgo de sufrir enfermedad cardiovascular y de colesterol (Gebauer *et al*, 2008; Ros, 2010). Contiene una alta proporción de ácidos grasos insaturados, además de un elevado contenido en micronutrientes, potasio, vitamina K y su consumo está relacionado con la mejora del perfil lipídico (Dreher, 2012; Costa *et al,* 2017).

La alergia a pistacho está menos estudiada que la de otros frutos de cáscara como la almendra, nuez o anacardo. Los trabajos publicados muestran que es más relevante en aquellas zonas dedicadas al cultivo del mismo, como Irán (Noorbakhsh *et al*, 2011). La prevalencia a pistacho se estima del 7% dentro de la alergia a frutos secos, en países como EEUU o Turquía (Weinberger & Sicherer, 2018). La reactividad cruzada entre anacardo y pistacho es frecuente dada su relación botánica y es común la aparición de reacciones clínicas graves o anafilaxis (Davoren & Peake, 2005; Noorbakhsh *et al,* 2011; Savvatianos *et al,* 2015; Elizur *et al,* 2017).

Proteínas alergénicas de anacardo y pistacho

Ana o 1 es una vicilina 7S identificada como alérgeno mayor o principal de anacardo, pues más del 50% de los pacientes alérgicos reaccionaron a la proteína Ana o 1 recombinante (Wang *et al*, 2002). La secuencia aminoacídica de Ana o 1 supera el 80% de identidad con la vicilina de pistacho, Pis v 3 y más del 50% con Cor a 11 de avellana. Los autores definieron los epítopos lineales de unión a IgE, que clasificaron en función de su reactividad frente a tres grupos de sueros. Recientemente, Reitsma y colaboradores (2016) han purificado e identificando Ana o 1 por MALDI-MS/MS como una glicoproteína de 50 kDa o de 100 kDa en forma de dímero. Ana o 2 es una legumina 11S compuesta de dos polipéptidos, uno más pesado y ácido de 30 kDa y una subunidad básica más pequeña de unos 21 kDa (Wang *et al*, 2003; Reitsma *et al*, 2016; Geiselhart *et al*, 2018). Se ha demostrado que la subunidad más grande de Ana o 2 requiere la asociación con la pequeña para la expresión de epítopos inmunoreactivos (Robotham *et al*, 2010; Reitsma *et al*, 2016). Ana o 3 es una albúmina 2S no glicosilada, que se procesa proteolíticamente en dos subunidades de 8 y 4 kDa, mantenidas por puentes disulfuro,

y muestran micro-heterogeneidad en los extremos N y C terminal (Robotham *et al,* 2005; Reitsma *et al,* 2016). Ana o 3 (rAna o 3) ha ganado interés en los últimos años como un marcador de alergia a anacardo muy específico comparado con el extracto completo de anacardo, pudiéndose convertir en una efectiva herramienta complementaria para el diagnóstico de la hipersensibilidad a este alimento (Lange *et al*, 2017; Van der Valk *et al*, 2017).

La albúmina Pis v 1 (de peso molecular estimado de 17 kDa en condiciones nativas) y la legumina Pis v 2 (50 kDa aproximadamente), fueron identificados y caracterizadas por Ahn et al (2009). Los autores describieron que la subunidades de 7 kDa de Pis v 1 y de 32 kDa de Pis v 2 reaccionaban con más del 50% de los sueros de pacientes empleados (alérgenos mayores). Pis v 1 comparte con su homóloga en anacardo, Ana o 3, un 64% de la secuencia aminoacídica, mientras que Pis v 2 muestra identidad de secuencia con Ana o 2 en un 48%. Pis v 5 es otra legumina que comparte un 79% de secuencia aminoacídica con su homólogo en anacardo. Pis v 3, por su parte, es un alérgeno mayor que pertenece a la familia de las vicilinas 7S. Se ha confirmado la existencia de reactividad cruzada entre Pis v 3 y Ana o 1, lo que explica en parte la co-sensibilización existente entre estos dos frutos secos (Willison *et al*, 2008). Por último, el único alérgeno descrito no perteneciente al grupo de las proteínas de reserva es Pis v 4, un alérgeno identificado como una superóxido dismutasa de manganeso (MnSOD, de aproximadamente 25 kDa). Este alérgeno podría ser responsable de reactividad cruzada con otras MnSOD de diferentes fuentes (Ayuso *et al,* 2007; Noorbakhsh *et al,* 2010).

2. PROCESADO DE ALIMENTOS DE ORIGEN VEGETAL

2.1. Efecto del procesado sobre la capacidad inmunoreactiva

Los alimentos son sometidos a una gran variedad de procesados para garantizar la seguridad y calidad alimentaria, mejorar sus propiedades organolépticas o prepararlos para su consumo. El procesado de alimentos provoca una serie de transformaciones e interacciones bioquímicas entre distintos compuestos y puede influir dramáticamente en las propiedades alergénicas de los mismos al destruir epítopos tanto conformacionales como lineales (Shriver & Yang, 2011; Rahaman *et al*, 2016). La eliminación de epítopos existentes o la formación de nuevos (neoalérgenos) son consecuencia habitual del cambio conformacional de las proteínas derivado del procesado (Jiménez-Saiz *et al*, 2015). La sensibilidad de cada proteína ante el procesado, su estructura, el tipo y condiciones de tratamiento aplicado así como la matriz alimentaria, van a determinar el alcance del procesado sobre su potencial alergénico (Maleki, 2004; Sathe & Sharma, 2009).

2.1.1. Tratamientos térmicos y de presión

Una de las reacciones bioquímicas derivadas del procesado térmico es la reacción entre grupos amino libre de proteínas con grupos aldehído de azúcares reductores, conocida como reacción de Maillard o glicación no enzimática de proteínas. Las reorganizaciones estructurales de estos compuestos (bases de Schiff) dan lugar a una serie de moléculas más estables conocidos como productos de Amadori. La reacción de Maillard contribuye a muchas de las propiedades organolépticas de ciertos alimentos procesados y ha demostrado influir considerablemente sobre la capacidad de los alérgenos para unir IgE (Maleki *et al*, 2000; Mills *et al*, 2009). La aplicación de ciertos tratamientos térmicos puede involucrar la pérdida de solubilidad de los alérgenos, debido a la formación de oligómeros, agregados o por reestructuración de proteínas o fragmentos proteicos (Maleki, 2004; Mills *et al*, 2009).

Los tratamientos térmicos (tostado, cocción, fritura, microondas) y de presión han mostrado eficacia a la hora de reducir la capacidad inmunoreactiva de los alimentos. Ciertos alérgenos de frutos secos, entre otros ingredientes alergénicos, son muy estables a la desnaturalización por calor. Algunos autores han descrito que el tratamiento térmico no reduce la alergenicidad de un determinado ingrediente o se requieren altas temperaturas para lograrlo (Venkatachalam *et al*, 2002; Su *et al*, 2004; Cuadrado *et al*, 2009). El tipo de calor aplicado (en condiciones secas o húmedas) afecta también a la estabilidad del potencial alergénico de las proteínas (Venkatachalam *et al*, 2006); así, el cacahuete tostado (calor seco) resulta más alergénico que su forma cruda, mientras que la cocción (condiciones húmedas) reduce la capacidad de las proteínas alergénicas para unir IgE (Maleki *et al*, 2000; Blanc *et al*, 2011; Petersen *et al*, 2014; Tao *et al*, 2016).

Los tratamientos que combinan calor y presión han resultado eficaces para reducir la capacidad de unir IgE en muchos alimentos de origen vegetal. Es el caso de la despresurización instantánea controlada (DIC®), en el que se aplican altas presiones y temperaturas durante pocos segundos (hasta 8 bar y 170^{o}C) (Allaf *et al*, 2000), y que ha demostrado ser útil para este fin en lupino, garbanzo o lenteja (Guillamón *et al,* 2008; Cuadrado *et al,* 2011). El tratamiento de calor y presión en autoclave, se ha aplicado sobre varios géneros de leguminosas, produciéndose una reducción significativa de las propiedades antigénicas de sus alérgenos (Álvarez-Álvarez *et al,* 2005; Venkatachalam *et al,* 2008; Cuadrado *et al,* 2009; Cabanillas *et al,* 2018). El autoclave también disminuye la capacidad de los alérgenos de nuez (138^{o}C/2.56 atm) y cacahuete (134^{o}C/2 atm) para unir IgE (Cabanillas *et al*, 2014; Bavaro *et al*, 2018). Este tipo de procesado puede inducir cambios conformacionales en las proteínas, agregación y/o desnaturalización de las mismas, que desembocan en una disminución de la capacidad para unir

IgE por parte de los epítopos (Rahaman *et al,* 2016). Algunos autores han determinado por dicroísmo circular la modificación y destrucción de gran parte de la estructura secundaria de proteínas alergénicas (como hélices α o láminas β) como consecuencia de determinados tipos de procesados (Cabanillas *et al*, 2012a; Luo *et al*, 2013).

Las altas presiones hidrostáticas (HHP) es un tratamiento no térmico empleado para preservar la calidad de los alimentos y obtener mejoras organolépticas, minimizando cambios de aroma, color u otros efectos potenciales derivados de la aplicación de calor (Shriver & Yang, 2011). La aplicación de altas presiones (100 MPa - 1 GPa) produce cambios estructurales en las proteínas en general, siendo por tanto una herramienta capaz de modificar la capacidad inmunoreactiva de las proteínas alergénicas (Somkuti & Smeller, 2013). Prieto y colaboradores (2014a) realizaron el análisis proteómico de las modificaciones de alérgenos de avellana provocados por el tratamiento con HHP. Sin embargo, los frutos de nuez tratada hasta 600 MPa de presión a 15^{o}C no produjeron cambios sustanciales en la capacidad de unir IgE (Cabanillas *et al*, 2014). El alérgeno de manzana Mal d 1 perdió sus propiedades alergénicas al aplicar 700 MPa y 115^{o}C de temperatura, mientras que el tratamiento no térmico en solitario no fue suficiente (Husband *et al*, 2011).

2.1.2. Hidrólisis enzimática

La digestión con proteasas es una herramienta empleada en industria alimentaria que permite mejorar el sabor de ciertos alimentos, su contenido nutricional o determinadas propiedades funcionales y complementariamente, se ha utilizado para reducir las propiedades reactivas frente a IgE (Tavano, 2013). La selección adecuada de las enzimas y la optimización de las condiciones de hidrólisis (temperatura, concentración de la enzima, pH, tiempo) son fundamentales para conseguir una reducción significativa del potencial alergénico (Venkatachalam *et al*, 2006; Lee *et al*, 2005; Cabanillas *et al*, 2010; Kulis *et al*, 2012; Mattison *et al*, 2014). Cabanillas y colaboradores (2012b) observaron que la digestión del extracto proteico de cacahuete tostado con la enzima Alcalasa, una endoproteasa, era más efectiva para reducir la unión a IgE que al hidrolizarlo con la exoproteasa Flavorzima. Venkatachalam *et al* (2006), por su parte, observaron niveles sustanciales de proteólisis únicamente a ratios específicos de concentración enzima:proteína en nuez pecana. Las fórmulas hidrolizadas por endo y exoproteasas de alimentos potencialmente alergénicos, como la leche de vaca, han sido comercializadas para su consumo por parte de individuos afectados de alergia alimentaria, al no incluir proteínas intactas (Clemente, 2000). Una de las características comunes de los alérgenos alimentarios es su elevada estabilidad a la digestión gastrointestinal, por lo que aplicar una pre-

hidrólisis enzimática de las proteínas resulta muy efectivo para reducir su inmunoreactividad, debido a la fragmentación de epítopos, fundamentalmente lineales (Rahaman *et al*, 2016). Un gran número de estudios han probado la susceptibilidad de los alérgenos alimentarios a la digestión gastrointestinal y el efecto de ésta sobre la alergenicidad, simulando las condiciones fisiológicas digestivas con mayor o menor grado de complejidad (Koppelman *et al*, 2010; Apostolovic *et al*, 2016; Bavaro *et al*, 2018). Algunos autores han analizado el hidrolizado proteico de frutos secos mediante digestión con las proteasas pepsina, tripsina/α-quimotripsina o pancreatina, evaluando la inmunoreactividad residual e incluso caracterizando los productos peptídicos resultantes (Venkatachalam *et al*, 2006; Cabanillas *et al*, 2010; Mattison *et al*, 2014; Korte *et al*, 2017).

2.2. Efecto del procesado sobre otras propiedades de los alimentos

Además de las proteínas, otros muchos compuestos nutricionalmente importantes pueden verse afectados por los tratamientos que se aplican sobre los alimentos (Verma *et al*, 2012). Los compuestos fenólicos, presentes en todas las plantas y asociados a propiedades preventivas frente a la inflamación o el cáncer, pueden sufrir cambios respecto a contenido y composición como consecuencia del procesado (Yao *et al,* 2004). La cocción, tratamiento habitual en la preparación de legumbres, provoca pérdidas en el contenido total de fenoles y de flavonoles de estos alimentos (Pedrosa *et al*, 2015; Siah *et al*, 2014). El tratamiento de DIC® reduce el contenido de azúcares, lectinas e inositol fosfatos en judías (Pedrosa *et al*, 2012). El contenido de fibra o de grasa son también susceptibles de verse modificados por determinados tratamientos (Gonçalves *et al*, 2010). La actividad antioxidante protege a las proteínas, lípidos y ácidos nucleicos del daño derivado de la liberación de radicales libres a partir del metabolismo celular (Cardador-Martínez *et al,* 2014). Esta propiedad también puede sufrir modificaciones tras el procesado de alimentos y verse aumentada o reducida en función de las condiciones de tratamiento y el alimento en cuestión (Chang *et al*, 2016).

Los frutos secos son una fuente rica en macro y micronutrientes, además de otros compuestos como fosfolípidos, tocoferoles, polifenoles, flavonoides y alcaloides, con un importante potencial antioxidante (Vinson & Cai, 2012). Su riqueza nutricional los convierte en un alimento muy saludable y, sumado a sus excepcionales características organolépticas, se proponen como parte esencial de una dieta óptima (Ros, 2010). Por los beneficios para la salud asociados a su consumo, las harinas de frutos secos, como ocurre con las de legumbres, arroz o ciertos tubérculos se han propuesto para su uso en industria alimentaria, además de como alternativas a las harinas de trigo en el desarrollo de formulaciones sin gluten (Juliano & Hicks,

1996; Alobo, 2003; De Vasconcelos *et al*, 2007; Ferreyra *et al*, 2007; Alobo *et al*, 2009; Aguilar-Raymundo & Velez-Ruiz, 2013). Las propiedades funcionales o tecnológicas de un alimento son aquellas propiedades físico-químicas, no nutricionales, que tienen repercusión sobre el comportamiento del alimento durante su preparación y almacenamiento (propiedades de hidratación, de asociación, emulsionantes y espumantes). Las proteínas y carbohidratos especialmente, pero también almidón, fibra y las interacciones entre todos ellos, serán responsables del comportamiento de los alimentos, condicionando la calidad y conservación del producto final (Bello Gutierrez, 2000). La capacidad de retención de agua, la viscosidad o la solubilidad son propiedades determinadas por el comportamiento de las macromoléculas frente al agua, mientras que la absorción de agua, retención de aceite o la capacidad gelificante están determinadas por interacciones iónicas, hidrofóbicas y enlaces covalentes. El procesado de los alimentos también puede llegar a tener un impacto importante sobre las propiedades funcionales y tecnológicas de harinas o extractos proteicos, lo que condicionaría su utilización en industria alimentaria como ingredientes innovadores. La influencia de una gran variedad de tratamientos sobre estas propiedades se ha estudiado ampliamente en legumbres y en menor medida, en frutos secos y otras semillas (Neto *et al*, 2001; Aguilera *et al*, 2009; Sharma *et al*, 2010; Aguilera *et al*, 2011; Ling *et al*, 2016).

3. ANÁLISIS DE ALÉRGENOS EN ALIMENTOS

3.1. Evaluación de la inmunoreactividad de los alimentos

Uno de los pasos iniciales para evaluar la alergenicidad de un alimento, como se ha indicado previamente, es identificar a los individuos alérgicos a través de una correcta caracterización clínica. El suero de estos pacientes y determinadas células del sistema inmunitario involucradas en el fenómeno de hipersensibilidad, serán necesarios para analizar la inmunoreactividad de los alimentos y de sus alérgenos. Esta evaluación del potencial alergénico de un ingrediente se realiza generalmente mediante métodos *in vitro, in vivo* e *in silico.*

Uno de los métodos *in vitro* estándar es el análisis de las proteínas alergénicas mediante electroforesis mono o bidimensional e inmunoblotting posterior por transferencia de las proteínas a una membrana de PVDF o nitrocelulosa, que se incuba con sueros IgE de pacientes alérgicos (Issaq & Veenstra, 2008). Permite identificar proteínas individuales inmunoreactivas determinando su capacidad de unir IgE de los sueros, aunque los alérgenos pueden estar alterados por la pérdida de epítopos conformacionales (Shriver & Yang, 2011). La técnica ELISA (de sus siglas en inglés *Enzyme-Linked Inmunosorbent Assay*) permite evaluar la alergenicidad

de un extracto proteico completo o un aislado proteico, gracias a su sensibilidad y relativa facilidad de uso (Rice & Lupo, 2014). En los últimos años, el diagnóstico por componentes (CRD, *component-resolved diagnosis*), que consiste en identificar las proteínas individuales a las que los pacientes están sensibilizados, ha ganado importancia en investigación clínica, empleando alérgenos nativos o recombinantes. Permite revelar los perfiles IgE individuales y obtener un examen más exhaustivo de posibles reacciones cruzadas (Treudler & Simon, 2013; Muraro *et al*, 2014b).

Como se ha indicado, el DBPCFC es la técnica "gold standard" para evaluar la alergenicidad de un alimento, pero es costoso y potencialmente peligroso para el paciente. Por ello, se han implantado de forma rutinaria ensayos que permiten detectar sensibilización a un alérgeno alimentario de un modo fiable y rápido, como las pruebas cutáneas (SPT, *skin prick test*), en las que se mide el tamaño de la pápula generada tras el contacto con el alérgeno. La unión de alérgenos alimentarios a anticuerpos IgE específicos anclados en las células efectoras conlleva la liberación de mediadores y la aparición de los síntomas de la alergia. El uso de modelos celulares de la respuesta alérgica permite detectar el potencial de los alérgenos para inducir una reacción mediada por IgE, a diferencia de los ensayos inmunológicos *in vitro* tradicionales. Estos modelos suelen ser basófilos/mastocitos humanos en los que se cuantifican marcadores de degranulación celular (BAT, *basophil activation test)* o líneas celulares de basófilos humanizadas (RBL, *rat basophil leukemia)*, con los que se realizan ensayos de liberación de mediadores de la respuesta alérgica (Eigenmann *et al*, 2013; Hoffmann *et al*, 2016). Estos modelos permiten incluso analizar una potencial aplicación en inmunoterapia (Sun *et al*, 2015). Las líneas RBL, que expresan los receptores humanos FcεRI, se han empleado para determinar cambios en la capacidad estimuladora de alérgenos tratados por distintos procesados (Shi *et al*, 2013; Cabanillas *et al*, 2014). En última instancia, los modelos animales son de gran utilidad para el estudio del desarrollo de la tolerancia a alérgenos alimentarios o para predecir la posible alergenicidad de nuevas proteínas (Helm *et al*, 2003; Kulis *et al*, 2012; Smaldini *et al*, 2012).

3.2. Etiquetado de alimentos

El reglamento (EU) nº 1169/2011 del Parlamento Europeo y del Consejo del 25 de octubre de 2011 sobre información alimentaria facilitada al consumidor pretende proteger la salud de los consumidores y asegurar su derecho a estar correctamente informados de los alimentos adquiridos (Journal of the European Union (22/11/2011)). La presencia de alérgenos y sustancias que provocan intolerancias alimentarias debe estar obligatoria y correctamente indicada en la etiqueta de los productos tanto envasados como no envasados, marcados

tipográficamente diferente a los ingredientes no peligrosos (www.aecosan.msssi.gob.es). Los 14 alérgenos de obligado etiquetado vienen representadas en el anexo II del mencionado reglamento (leche de vaca, huevo, frutos de cáscara, soja, cacahuete, cereales con gluten, pescado, moluscos, crustáceos, apio, mostaza, sésamo, lupino y dióxido de azufre/sulfitos).

La dosis umbral es la cantidad mínima de un ingrediente o estímulo que desencadena una respuesta o reacción; en el caso de los alérgenos es la concentración mínima capaz de desencadenar la sensibilización (en la primera fase de contacto) y/o reacción alérgica (en la fase sintomática) (Crevel *et al*, 2008). Se han propuesto dosis umbral para riesgos alimentarios como sulfitos, gluten y otros agentes microbiológicos o químicos, pero en el caso de proteínas alergénicas no se ha establecido oficialmente. La determinación de la concentración máxima de un alimento que no produzca efectos adversos es muy compleja, pues hasta la más mínima cantidad de un alérgeno puede provocar síntomas severos en individuos con un elevado nivel de sensibilidad (Crevel *et al*, 2008; Taylor *et al*, 2010). En consecuencia, la industria alimentaria se encuentra ante la situación de tener que emplear frecuentemente el ambiguo mensaje "puede contener" en la etiqueta de sus productos (Crevel *et al*, 2008; Siragakis & Kizis, 2014).

La industria alimentaria juega un papel esencial en la identificación, procesado y comercialización de los alimentos. La presencia de alérgenos no deseados en un alimento puede deberse a 1) contaminación accidental con un ingrediente potencialmente alergénico en la fábrica o en empresas colaboradoras 2) contaminación no declarada de un ingrediente potencialmente alergénico en alguno de los puntos de fabricación o 3) sustitución fraudulenta de uno de los ingredientes del alimento por otro de menor valor o precio. Para proteger la salud de los individuos sensibilizados es indispensable un correcto etiquetado de los alimentos, completo y sencillo de visualizar por el consumidor interesado. Para conseguir este propósito resulta necesario desarrollar métodos analíticos de detección de alérgenos específicos, sensibles y reproducibles (De la Cruz *et al*, 2017).

Las técnicas inmunológicas y las moleculares, basadas en la detección de proteínas y ADN respectivamente, son las más empleadas actualmente por ser rápidas y efectivas (Prado *et al*, 2015). Se están desarrollando técnicas alternativas para la identificación y detección de alérgenos, con el propósito final de miniaturizar la tecnología para hacerla más aplicable en el sector del análisis de alimentos y seguridad alimentaria. Para poder desarrollar un método cuantitativo validado se requieren materiales de referencia certificados (CRMs, Certified Reference Materials). Si bien existen materiales de referencia comercialmente disponibles para muchos alérgenos alimentarios, los resultados obtenidos con ellos no son comparables porque no son certificados (EFSA NDA Panel, 2014). No existen CRMs disponibles para validar métodos

de detección de alérgenos a día de hoy, a excepción de cacahuete en métodos inmunológicos, producido por el IRMM (Institute for Reference Materials and Measurements, Geel, Bélgica).

3.3. Métodos de detección de alérgenos basados en proteínas

3.3.1. ELISA y Dispositivos de flujo lateral

Los métodos inmunológicos son aquellos en los que la diana es una o más proteínas indicadoras de la presencia de un determinado ingrediente causante de una reacción alérgica. Los métodos más utilizados en industria alimentaria son el ELISA, que emplea anticuerpos IgG específicos obtenidos a partir un animal inmunizado y los dispositivos de flujo lateral (DFL/LFD, *lateral flow devices*) (Rice & Lupo, 2014; Prado *et al*, 2015). La técnica basada en DFL es una técnica cualitativa, barata y muy sencilla de utilizar, alternativa al ELISA. Implican el flujo por capilaridad de la muestra líquida problema a través de una membrana de nitrocelulosa hasta contactar con los anticuerpos inmovilizados en la misma (Bahadır & Sezgintürk, 2016). Los inconvenientes o limitaciones más importantes que pueden presentar los métodos basados en proteínas son:

- Cambio en la conformación o estructura del alérgeno/antígeno, consecuencia del procesado tecnológico, afectando a la interacción con el anticuerpo
- Solubilidad deficiente de las proteínas a partir de la matriz alimentaria
- Extracción insuficiente de las proteínas a partir de la matriz alimentaria como consecuencia de un tratamiento tecnológico aplicado
- Especificidad insuficiente por reactividad cruzada entre alérgenos

3.3.2. Espectrometría de masas

La estrategia más empleada para el análisis de proteínas alergénicas mediante espectrometría de masas requiere un paso de separación de las proteínas (basado en electroforesis y/o cromatografía líquida (LC)) seguido del análisis por espectrometría de masas (MS), obteniendo datos de identificación de proteínas y péptidos de la muestra (Cunsolo *et al*, 2014). La estrategia *bottom-up* emplea digestión de las proteínas a péptidos mediante proteasas para el análisis subsecuente de MS, mientras que en la estrategia *top-down* se analizan proteínas intactas. Esta técnica permite detectar, cuantificar y/o identificar precisa e inequívocamente proteínas (García-Cañas *et al*, 2012) y está cobrando interés en el ámbito de calidad y seguridad alimentaria, como en detección de alérgenos (Chassaigne *et al*, 2007; Monaci & Visconti, 2009; Prado *et al*, 2015).

3.3.3. Biosensores de proteínas

Un biosensor es un dispositivo que permite el reconocimiento biológico de dos moléculas para un bioanálisis concreto (Kumar, 2008). Una de sus principales ventajas, que los convierte en una interesante alternativa a otras técnicas habituales, es la minimización en tiempo de preparación de la muestra (pre-análisis), lo que permite el estudio *in situ* en la industria alimentaria o en los laboratorios de control (Kumar, 2008). Los biosensores se pueden clasificar en función del bioreceptor o del transductor empleado (Figura 5).

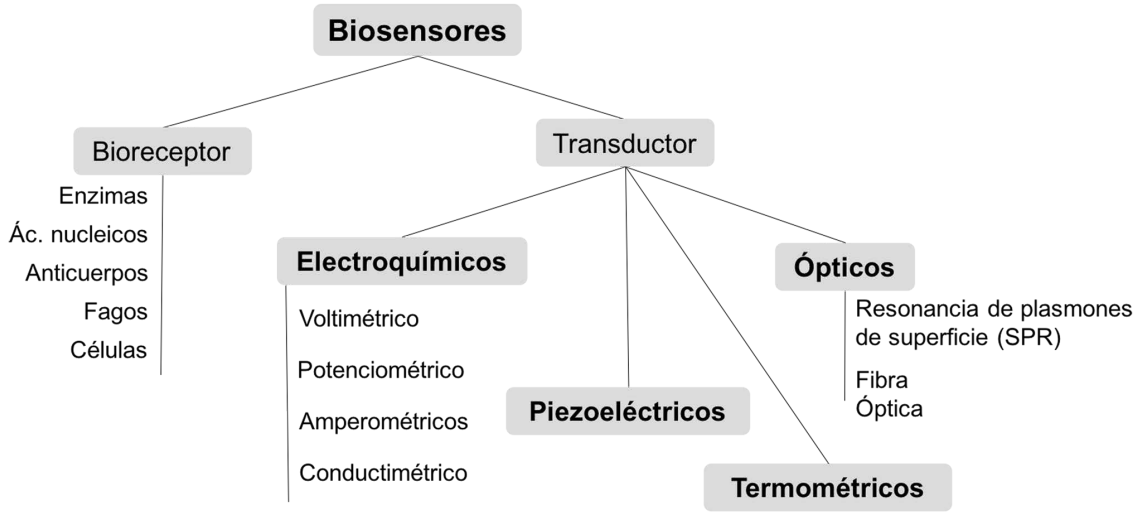


Figura 5. Clasificación de los biosensores según el bioreceptor o el transductor. Adaptación de (Velusamy *et al*, 2010).

Los biosensores ópticos se basan en la generación de ondas evanescentes, en los que se mide la variación del índice de refracción en la superficie del sensor cuando se produce la interacción receptor-analito. Los biosensores de resonancia de plasmones superficiales (SPR) son los más reseñables dentro de este grupo (Homola, 2003). La proteína Ara h 1 de cacahuete o la α-caseína de leche de vaca se han detectado con este tipo de biosensores con elevada sensibilidad (Pollet *et al*, 2011). Los biosensores electroquímicos se basan en la detección de cambios en la conductancia asociados al ambiente iónico (conductimétricos), potencial (voltimétricos), actividad de iones en una reacción electroquímica (potenciométricos) o reacciones de oxidación/reducción (amperométricos) (Figura 5). Recientemente se han desarrollado biosensores amperométricos basados en partículas magnéticas que permite la detección de la proteína α-lactoalbúmina o Ara h 2 (Ruiz-Valdepeñas *et al*, 2016a, 2016b). Los biosensores piezoeléctricos se basan en materiales con el mismo nombre que poseen la capacidad de acumular carga eléctrica cuando se somete a tensión mecánica (Thakur & Ragavan, 2013; Alves *et al*, 2016). Xiulan y colaboradores (2010) desarrollaron un sistema de detección rápido con un inmunosensor de este tipo del alérgeno alimentario Pen a 1, de un tipo de

camarón de agua dulce. El uso de los biosensores termométricos o calorimétricos en control de calidad y detección de alérgenos es menos frecuente que los biosensores ya mencionados (Ramanathan & Danielsson, 2001).

3.4. Métodos de detección de alérgenos basados en ADN

El uso de métodos moleculares basados en la detección de secuencias específicas de ADN está extendido a múltiples aplicaciones, incluida la autenticación de ingredientes y control de calidad, detección de patógenos, de organismos modificados genéticamente (OMG) y de alérgenos alimentarios como alternativa a los métodos inmunológicos (De la Cruz *et al*, 2017). Se trata de una metodología que permite la detección de ingredientes alergénicos de forma rápida, específica y sensible. Se consideran métodos indirectos al detectarse una secuencia de ADN que evidencia la presencia del ingrediente alergénico, en lugar de la molécula responsable de la respuesta alérgica. Una de las principales ventajas que presentan estos métodos radica en que, frente a determinados procesados, el ADN se ve menos afectado que las proteínas, cuyos cambios en la solubilidad y/o conformación tras el procesamiento, pueden tener un impacto negativo en la posterior detección de las mismas (Bergerová *et al*, 2010; Gryson, 2010). Los métodos basados en la detección de ácidos nucleicos se fundamentan, principalmente, en la amplificación de fragmentos específicos de ADN mediante la reacción en cadena de la polimerasa (PCR) ofreciendo resultados cualitativos de presencia/ausencia de alérgeno (PCR de punto final) o cuantitativos (PCR en tiempo real).

Los métodos de detección de ADN en muestras alimentarias comprenden tres etapas. La primera es la extracción y purificación de ADN de buena calidad para que pueda ser detectado; en muestras alimentarias complejas es habitual la presencia de una amplia variedad de compuestos que pueden co-extraerse junto con el ADN y en su caso interferir en la reacción de amplificación (Costa *et al*, 2015). Los inhibidores de la reacción de PCR son un grupo amplio y heterogéneo de sustancias cuya presencia en la reacción puede dar lugar a la obtención de resultados inexactos o falsos negativos (Schrader *et al*, 2012). El segundo paso es el de amplificación de secuencias de ADN específicas de la especie o especies de interés y por último, la detección del ADN sintetizado.

3.4.1. Reacción en cadena de la polimerasa

La PCR consiste en una serie de ciclos con tres etapas de calentamiento y enfriamiento a temperaturas definidas. La primera es la desnaturalización a 94 - 95 ^{o}C, en la que el ADN se desnaturaliza, separándose las hebras complementarias. A continuación, la temperatura

disminuye hasta aquella que es óptima para que los cebadores o sondas se unan de forma específica a sus complementarias en el ADN molde (hibridación o *annealing* en inglés). En la última etapa, de extensión o amplificación, la temperatura asciende de nuevo hasta 70-72^{o}C óptima para el funcionamiento de la ADN polimerasa, que sintetiza la nueva cadena a partir de los cebadores. Este ciclo completo se repite 30-40 veces, obteniéndose una acumulación exponencial del ADN diana al final de la reacción.

La PCR de punto final es una técnica cualitativa en la que los productos amplificados se detectan al final de la reacción, habitualmente mediante geles de agarosa y tinción con agentes intercalantes como el bromuro de etidio, que permiten la visualización del resultado. La técnica de PCR de punto final ha permitido la detección cualitativa de frutos secos como pistacho, cacahuete, avellana o nuez en distintas matrices, con diferente sensibilidad (límite de detección, abreviado LOD del inglés) (Herman *et al,* 2003; Watanabe et al, 2006; Barbieri & Frigeri, 2006; Yano et al, 2007). Además del formato más convencional la PCR tiene el potencial de amplificar y detectar más de una secuencia simultáneamente en una matriz alimentaria, mediante el formato múltiple o *multiplex.* Mediante esta técnica se ha detectado nuez de Brasil y nuez pecana en una única reacción de PCR de punto final (Hubalkova & Rencova, 2011). La principal ventaja del formato múltiple es el ahorro de tiempo y costes; sin embargo, la sensibilidad del ensayo suele reducirse con respecto al formato de detección de un único alérgeno por PCR, pues requiere amplificación diferencial de las distintas secuencias diana (Pafundo *et al*, 2010).

Algunos de estos inconvenientes se pueden salvar con una variante de la técnica denominada amplificación de sondas dependiente de ligando, o LPA de sus siglas en inglés (*Ligation-dependent probe amplification)*. El LPA o MLPA (en *multiplex*) se basa en la amplificación de productos que resultan de una primera reacción de unión de hemi-sondas con la zona de interés, que posteriormente son ligadas y amplificadas por PCR. Cada hemi-sonda contiene uno de los cebadores, el directo o el inverso, siendo uno de ellos el que incorpora un fluoróforo que permite la detección. Además de los cebadores, las sondas incluyen otro oligonucleótido capaz de hibridar con la secuencia diana, así como una secuencia de relleno de tamaño variable (*stuffer* sequence) presente en una de las hemi-sondas y que permite modificar la longitud de la misma en función del experimento. El resultado se visualiza mediante análisis de fragmentos por electroforesis capilar (Schouten *et al*, 2002; De la Cruz *et al*, 2017). Es una técnica que se ha empleado en biomedicina para el diagnóstico simultáneo de distintas anomalías genéticas (Gatta *et al*, 2005). En control de calidad y detección de alérgenos en alimentos, se han desarrollado métodos de detección simultánea, de hasta 10 especies, basados en MLPA (Mustorp *et al*, 2011; García-García *et al*, 2018).

3.4.2. PCR en tiempo real

La PCR en tiempo real (RT-PCR, *real time PCR*) es un tipo de PCR que permite la visualización o monitorización de la amplificación de manera continua (a tiempo real) y proporciona mayor sensibilidad al utilizarse fluorescencia para detectar el producto de la amplificación. La cantidad de fluorescencia obtenida será proporcional a la cantidad de producto amplificado, lo que posibilita la cuantificación. Aunque más sensible, es una técnica más cara y compleja que la PCR de punto final (Valasek & Repa, 2005).

Diseño de cebadores

El objetivo de desarrollar un método basado en PCR es conseguir un sistema sensible y específico. La selección de la secuencia diana y el diseño de las sondas para la amplificación de la misma es uno de los pasos más relevantes a la hora de desarrollar un protocolo o método de detección de alérgenos basado en PCR, ya sea de punto final o de tiempo real. Las secuencias seleccionadas como diana pueden ser las codificantes para la proteína alergénica (generalmente genes de una o pocas copias) u otras secuencias de la especie potencialmente alergénica. En este último grupo las más utilizadas son las secuencias ITS (de las siglas en inglés *Internal Transcribed Spacer*), secuencias multicopia del genoma nuclear. También se han utilizado otras secuencias diana situadas en el genoma de los plastos o de las mitocondrias. La secuencia de ADN seleccionada como diana va a ser responsable en gran medida de los resultados de sensibilidad y especificidad, el sistema de detección es generalmente más sensible cuando se emplean como diana secuencias multicopia (Prado *et al*, 2012). Los genes de copia única como pueden ser las secuencias codificantes de alérgenos se recomiendan para desarrollar métodos cuantitativos, y suelen ser más específicos (Prado *et al,* 2012*)*. La disponibilidad de las bases de datos de secuencias permite la selección de la diana más adecuada según el objetivo que se persiga. Una vez seleccionado el gen o secuencia a amplificar en la especie de interés, se realiza una búsqueda mediante el programa BLAST (de las siglas inglesas *Basic Local Alignment Search Too*l, blastn*)* para identificar genes homólogos en otras especies incluidos en la base de datos. Las secuencias que muestran suficiente identidad se consideran homólogas y se comparan entre sí mediante alineamiento múltiple (Iniesto *et al*, 2013; Prieto *et al*, 2014b). Mediante este análisis, se selecciona la región potencialmente adecuada para diseñar los cebadores/sondas: una zona de la secuencia del gen diana que muestre variabilidad interespecífica y a su vez esté conservada entre variedades o cultivares de la especie a detectar.

Los algoritmos disponibles para diseñar los cebadores son herramientas útiles para el diseño inicial de los mismos ya que permiten predecir tanto la temperatura de fusión de los

oligonucleótidos (Tm) como la formación de posibles estructuras secundarias o dímeros que puedan afectar a la eficiencia de la reacción (Apte & Daniel, 2009). En la práctica la temperatura óptima debe ser determinada experimentalmente, ya que la hibridación deficiente de los cebadores puede afectar a la calidad del ensayo. Del mismo modo, la especificidad también debe evaluarse de forma experimental, ya que las precauciones tomadas *in silico* para el diseño de las sondas no siempre aseguran que el ensayo de lugar a los resultados esperados (Bustin *et al,* 2009).

Químicas de detección por PCR en tiempo real

La química más simple y barata para detectar los productos amplificados es la basada en moléculas intercalantes fluorescentes, como el SYBR Green, que al unirse a la doble hebra de ADN emiten fluorescencia. Las curvas de disociación, propias de tecnologías de detección en las cuales el fluoróforo permanece asociado al amplicón, permiten identificar los fragmentos específicos en base a la temperatura de disociación característica del amplicón (Ririe *et al*, 1997). Suelen representarse como el cambio de fluorescencia con respecto al cambio de temperatura frente a la temperatura (ΔFΔT vs temperatura), obteniéndose un gráfico de la dinámica de disociación que permite discriminar la señal específica del amplicón de otros productos inespecíficos (Klein *et al*, 2002).

En las técnicas de detección por RT-PCR basadas en la química de sondas se emplean oligonucleótidos de ADN acoplados a moléculas fluorescentes. Confieren alta especificidad, pues en este caso se emplean dos cebadores y una sonda que, en función de la longitud, pueden llegar cubrir toda la secuencia diana. En el extremo de la sonda incluyen un fluoróforo unido covalentemente denominado donador (*reporter* en inglés) y un agente bloqueador en el otro extremo (*quencher*) (Figura 6). Cuando ambas moléculas se encuentran próximas físicamente, el bloqueador impide la emisión de fluorescencia por parte del donador por un mecanismo conocido como transferencia de energía de resonancia de Föster (FRET), que explica el solapamiento de la excitación de un fluoróforo donador por el espectro de emisión del bloqueador (Didenko, 2001).

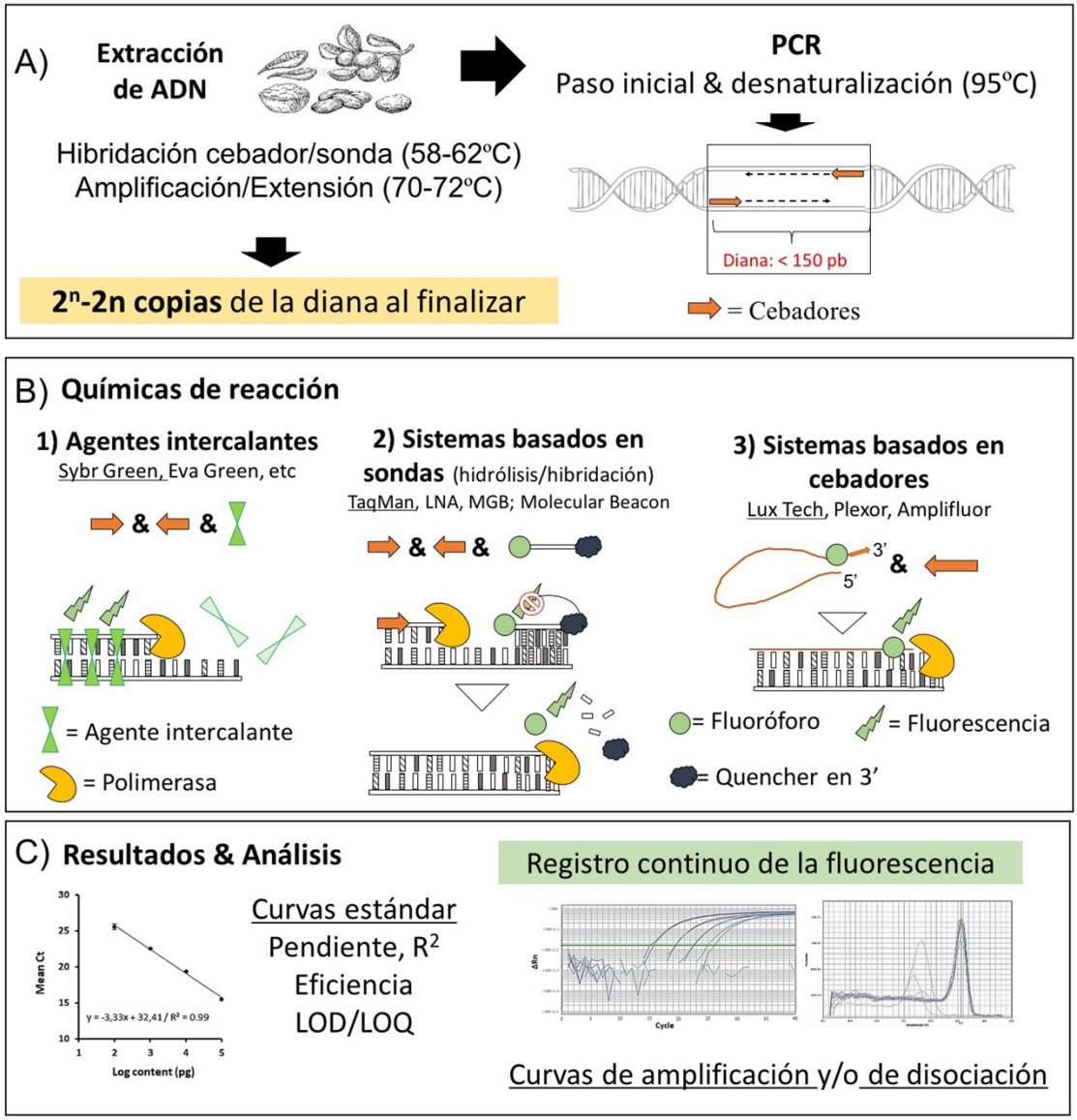


Figura 6. Representación esquemática de la reacción de PCR a tiempo real A) Pasos generales de la PCR, B) Químicas de reacción para PCR en tiempo real. Se subraya la química que es representada, C) Análisis de los datos obtenidos tras la reacción de la PCR en tiempo real.

Las sondas fluorescentes se clasifican generalmente en dos tipos: de hidrólisis y de hibridación. En el primer tipo, la actividad nucleasa 5' de la polimerasa provocará la hidrólisis de la sonda al alcanzarla durante la fase de extensión. Esta ruptura liberará al fluoróforo de la actividad bloqueadora, emitiendo la fluorescencia que se recoge de manera continua. La tecnología TaqMan es la más clásica y conocida dentro de las sondas de hidrólisis. Estas sondas tienen una longitud de 15-30 nt y requieren una temperatura de hibridación de 8-10ºC por encima de la Tm de los cebadores. De esta forma, la sonda se une a la diana antes que éstos, asegurando la detección de la señal cuando se extiende la molécula de ADN a partir de los cebadores. En la tabla 2 se recopilan trabajos en los que se desarrollan sistemas de detección de ADN de frutos secos mediante RT-PCR, incluyendo los basados en sondas TaqMan. Las sondas

de unión al surco menor o MGB (del inglés Minor Groove Binding) incorporan en el extremo 3' un grupo no fluorescente (NFQ), que proporciona resultados teóricamente más precisos y con menor ruido de fondo (Yao *et al*, 2006). Su estructura permite una unión más estable con la hebra complementaria e incrementa la temperatura de hibridación. Con el empleo de las sondas MGB se han detectado secuencias codificantes de un alérgeno mayor de pescado, la parvalbúmina (Sun *et al*, 2009). Otras sondas de hidrólisis modificadas son las basadas en ácido nucleico bloqueado LNA (de sus siglas en inglés, Locked Nucleic Acid). Estas sondas, pese a su pequeño tamaño de 8-9 nucleótidos, presentan una elevada temperatura de hibridación. Los LNA son análogos de nucleótidos que, comparado con los desoxirrubonucleótidos estándares, tienen una fuerza de unión mayor a la secuencia complementaria gracias a un puente adicional que une el oxígeno de la posición 2' y el carbono de la posición 4' de la ribosa, lo que "bloquea" su estructura (Figura 7). La tecnología LNA asegura que un cambio de un solo nucleótido causa inestabilidad de la sonda, confiriendo por tanto, alta especificidad (Echwald, Andreasen & Mourizen, 2016).

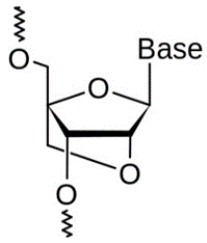


Figura 7. Estructura química de un monómero de LNA.

Las sondas de hibridación, como las Molecular Bacon, suelen tener una conformación de horquilla que mantiene próximos los dos extremos de la sonda en los que se encuentran los fluoróforos/bloqueador, evitando la emisión de fluorescencia. Cuando se produce la hibridación de este oligonucleótido con la secuencia complementaria se separan físicamente los extremos, liberándose la fluorescencia (Gašparič *et al*, 2010). Este sistema ha sido empleado para la identificación de OMG (Andersen *et al*, 2006).

La química basada en el uso de cebadores, como las tecnologías *Lux*, *Amplifluor* o *Plexor*, está menos extendida (Gašparič *et al*, 2010). En la tecnología *Lux,* el cebador directo tiene un fluoróforo adherido al extremo 3' que emite fluorescencia al hibridar con la secuencia diana, cuando se pierde la estructura en horquilla. La tecnología *Amplifluor* emplea tres cebadores, dos de ellos específicos y otro universal marcado en sus dos extremos, con estructura de horquilla. Se ha evaluado su uso para detección de OMG (Hernández et al, 2004; Rodríguez-Lázaro et al, 2004). En la tecnología *Plexor*, a diferencia de las otras químicas, la fluorescencia que se recoge

va descendiendo a medida que aumenta la cantidad de producto de PCR (Gašparič *et al*, 2010). Recientemente se ha publicado un sistema de detección de albaricoque en mazapanes que declaran almendra (fraude alimentario) basado en esta tecnología (Schelm *et al*, 2017).

Las curvas de amplificación representan la emisión de fluorescencia relativa a medida que avanzan los ciclos de la reacción (Figura 6). Generalmente, la fluorescencia emitida en los primeros ciclos de la reacción se establece como señal base (*baseline*), cuando los cambios de fluorescencia observados son pequeños, lo que permite eliminar el ruido de fondo. Con respecto a ésta, se calcula el umbral de fluorescencia (threshold), que es el nivel de señal que refleja un incremento estadísticamente significativo con respecto a la señal calculada como línea base. Es importante que ambas señales sean establecidas correctamente para que los valores posteriores de ciclo umbral (Ct, de cycle threshold) sean determinados con precisión. El Ct se define como el ciclo en el cual la señal fluorescente emitida alcanza el valor umbral (threshold), está inversamente relacionado con la cantidad de molde y sirve como base para la cuantificación absoluta o relativa del ADN.

La PCR en tiempo real, empleando distintas químicas de detección y seleccionando distintos tipos de secuencias diana, ha sido utilizada como técnica de detección de ADN de distintos alérgenos alimentarios(Hupfer et al, 2007; Demmel et al, 2008; Hildebrandt & Garber, 2010; Siegel et al, 2012; Graziano et al, 2017). El formato *multiplex* también se ha desarrollado para RT-PCR. Köppel y colaboradores (2009) llegaron a detectar simultáneamente cacahuete, avellana, apio y soja. En otra reacción equivalente, los mismos autores detectaron leche, huevo, almendra y sésamo, todos ellos con un límite de detección de 50 ppm.

En la tabla 2 se incluyen los principales trabajos en los que se describen métodos de PCR en tiempo real para la detección de frutos de cáscara.

Tabla 2. Métodos de detección de alérgenos de frutos de cáscara por PCR en tiempo real.

Método	Diana	LOD (mg/kg)	Referencia
Almendra			
TaqMan	ITS 1	0.1	López-calleja *et al*, 2014a
SYBR Green	Pru du 1	1	Prieto *et al*, 2014
	Pru du 3	5	
TaqMan	Pru du 6	50	Costa et al, 2013a
EvaGreen+HRM	Pru du 5	50	Costa *et al*, 2012
TaqMan	nsLTP	5	Roder *et al*, 2011
SYBR Green	Pru du 1	N.D.	Pafundo *et al*, 2009
TaqMan	Pru av 1	50	Köppel *et al,* 2009[1]
Anacardo			
LNA	Ana o 1	10	Sanchiz *et al* 2018*
TaqMan	Ana o 1	10	
TaqMan	ITS 1	0.1	López-Calleja *et al*, 2015a
TaqMan	Ana o 3	5	Köppel *et al*, 2012[1]
SYBRGreen	Ana o 1	0.5 pg	Pafundo *et al*, 2010[1]
TaqMan	Ana o 3	2	Ehlert *et al*, 2008
TaqMan	Ana o 3	100	Piknová & Kuchta, 2007
TaqMan	Ana o 3	100	Brzezinski, 2006
Avellana			
TaqMan	hsp 1	50	Costa *et al*, 2014
TaqMan	ITS 1	0.1	López-Calleja *et al*, 2013
SYBR Green	Cor a 9	1	Iniesto *et al*, 2013
TaqMan	Cor a 1	5	Köppel *et al*, 2012[1]
SYBR Green	Cor a 1	10	D'Andrea *et al*, 2011
	Cor a 8	10	
	Cor a 14	10	
TaqMan	Cor a 1	50	Köppel *et al,* 2009[1]
TaqMan	hsp 1	100	Piknová *et al*, 2008
TaqMan	Cor a 1	0.1 ng	Arlorio *et al*, 2007
Nuez			
SYBRGreen	Jug r 3	100	Linacero et al., 2016*
TaqMan	ITS 1	0.1	López-Calleja *et al*, 2015b
TaqMan	Jug r 3	50	Costa *et al*, 2013b
TaqMan	Jug r 2	20	Köppel *et al*, 2012[1]
SYBRGreenER	Jug r 1	0.5 pg	Pafundo *et al*, 2010[1]
TaqMan	Jug r 2	10	Wang *et al*, 2009
TaqMan	Jug r 2	100	Brežná *et al*, 2006
Nuez de Brasil			
TaqMan	Ber e 1	2.5	De la Cruz *et al*, 2013
TaqMan	Ber e 1	5	Roder *et al*, 2010
TaqMan	Ber e 2	1000	Brežná *et al*, 2010
Nuez de Macadamia			
TaqMan	ITS 1	0.1	López-Calleja *et al,* 2015a
TaqMan	Vicilina	200	Brežná *et al*, 2009
Nuez pecana			
TaqMan	ITS 1	0.1	López-Calleja *et al*, 2015b
TaqMan	Car i 2	100	Brežná & Kuchta, 2008
Piñón			
TaqMan	tRNA-Leu	0.1	Garino *et al*, 2016
Pistacho			
LNA	Pis v 1	10	Sanchiz *et al*, 2017*
SYBR Green	Pis v 1	100	
TaqMan	ITS 1	0.1	López-Calleja *et al,* 2014b
TaqMan	ITS	4	Brežná *et al*, 2008

*****Trabajos presentados en esta tesis doctoral; N.D. no declarado; [1] Sistema *multiplex*

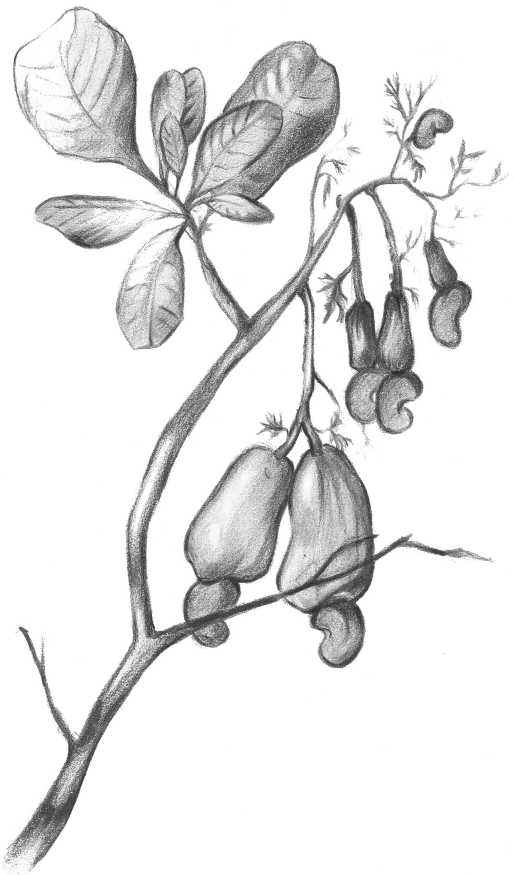
Como se ha comentado previamente en esta Introducción, los alimentos son sometidos a una amplia variedad de procesados. Pese a la estabilidad que presenta el ADN con respecto a

las proteínas, ciertos tratamientos pueden llegar a fragmentar o degradar el ADN (Bauer *et al*, 2003; Gryson, 2010). La mayoría de los trabajos desarrollan métodos de detección de secuencias de ADN evaluadas en mezclas crudas y sin tratar; sin embargo, algunos autores han resaltado la conveniencia de analizar y determinar el efecto del procesado sobre la detección de la secuencia diana por PCR, sea de alérgenos (Platteau *et al*, 2011; Iniesto *et al*, 2013; Prieto *et al*, 2014b) o de OMG (Bergerová *et al*, 2010; Tian *et al*, 2014). En función del tratamiento aplicado y del alimento analizado, la influencia sobre la detección de la secuencia diana puede diferir. Hird y colaboradores (2006) demostraron que determinados procesados térmicos pueden comprometer la detección de alimentos cárnicos debido a la rotura del ADN; sin embargo, otros tratamientos como las altas presiones hidrostáticas (HHP) no influyen en la detección sensible de trazas de almendra (Prieto *et al,* 2014).

3.4.3. Biosensores de ADN

Los biosensores de ADN o genosensores tienen ácidos nucleicos como bioreceptor del dispositivo (Figura 5). Su uso es menos frecuente que los de anticuerpos o enzimas, pero se han desarrollado genosensores para la detección de secuencias procedentes de algunos alérgenos alimentarios, incluyendo frutos secos como avellana y cacahuete (Tortajada-Genaro *et al*, 2012). Wang y colaboradores (2011) desarrollaron un genosensor óptico que permite la detección simultánea de varios alérgenos, gracias a cambios en la coloración del espectro visible. La unión de la técnica PCR con biosensores electroquímicos de ADN permite combinar las ventajas que presentan ambas metodologías, la alta selectividad de los métodos basados en la amplificación del ADN con la posibilidad de simplificar el proceso que ofrecen los biosensores (Ruiz-Valdepeñas *et al*, 2018). Bettazzi *et al* (2008) diseñaron un sistema electroquímico en combinación con PCR para identificar Cor a 1 de avellana en alimentos con un LOD de 0.1 nM. Posteriormente, mediante un genosensor amperométrico que incluye una reacción de PCR (*Express PCR)* se ha detectado la secuencia diana Cor a 9 de avellana (LOD 0.72 pM) (Ruiz-Valdepeñas *et al*, 2017). En trigo, se ha detectado una diana específica de gliadina mediante un genosensor electroquímico (Martín-Fernández *et al*, 2015). Los dispositivos piezoeléctricos basados en ADN también han resultado útiles y efectivos en seguridad alimentaria, para la detección de marcadores genéticos de patógenos como *E. coli* O157:H7 u hongos productores de micotoxinas (Kumar *et al*, 2012).

OBJETIVOS



Los objetivos generales de la presente Tesis Doctoral son:

1) Analizar el efecto del procesado térmico, de presión y enzimático sobre la inmunoreactividad de anacardo y pistacho
2) Desarrollar un sistema de detección, basado en PCR en tiempo real, de frutos secos en alimentos procesados

Para la consecución de estos objetivos generales, se establecen los siguientes objetivos específicos:

- Estudiar la influencia del tratamiento térmico y de presión (cocción y autoclave) en la reactividad frente a IgE de anacardo y pistacho
- Evaluar el efecto del tratamiento enzimático en combinación con el procesado térmico y de presión sobre la inmunoreactividad de anacardo y pistacho
- Analizar el impacto del procesado térmico y de presión sobre el contenido de compuestos fenólicos, actividad antioxidante y propiedades tecno-funcionales de las harinas de anacardo, pistacho y castaña
- Diseñar cebadores y/o sondas específicos, a partir de las secuencias de genes codificantes de alérgenos, en nuez, pistacho y anacardo
- Evaluar la sensibilidad, especificidad y eficiencia de los métodos de PCR en tiempo real con distintas químicas de detección
- Analizar el efecto de los tratamientos térmicos y de presión (cocción, HHP o autoclave), sobre la detección de nuez, pistacho o anacardo
- Evaluar la aplicabilidad de los métodos de PCR en tiempo real desarrollados para detectar la presencia de trazas de nuez, pistacho o anacardo en alimentos procesados

RESULTADOS

BLOQUE I

Efecto del procesado térmico, de presión y enzimático sobre alérgenos de pistacho y anacardo.

ARTÍCULO 1.

EFECTOS DEL PROCESADO TÉRMICO EN LA REACTIVIDAD FRENTE A IGE DE ANACARDO Y PISTACHO

THERMAL PROCESSING EFFECTS ON THE IGE-REACTIVITY OF CASHEW AND PISTACHIO

El procesado térmico puede modificar la estructura y función de las proteínas alergénicas y alterar así su alergenicidad. Este trabajo tiene como objetivo analizar la influencia del tratamiento térmico en la reactividad frente a IgE de anacardo y pistacho. La inmunodetección por western blot y ELISA se ha complementado con pruebas cutáneas tipo "Prick" (SPT) y ensayo de liberación de mediadores para determinar la capacidad de unir IgE (*cross-linking)*. El procesado térmico disminuyó las propiedades de unión a IgE en ambos frutos secos, especialmente tras el tratamiento de calor y presión. El tamaño de la pápula se redujo considerablemente tras la aplicación de las muestras térmicamente tratadas. En anacardo, las muestras tratadas por calor y presión todavía retuvieron cierta capacidad de unir IgE y degranular basófilos, sin embargo, esta capacidad se veía muy reducida comparado con el anacardo sin tratar. En pistacho, la degranulación de los basófilos se redujo considerablemente después de la estimulación con el extracto más severamente tratado por calor y presión. La cocción mostró resultados más variables, sin embargo, este tratamiento aplicado sobre ambos frutos secos durante 60 minutos, produjo un importante descenso en la degranulación de los basófilos.

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Thermal processing effects on the IgE-reactivity of cashew and pistachio

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ABSTRACT

Thermal processing can modify the structure and function of food proteins and may alter their allergenicity. This work aimed to elucidate the influence of moist thermal treatments on the IgE-reactivity of cashew and pistachio. IgE-western blot and IgE-ELISA were complemented by Skin Prick Testing (SPT) and mediator release assay to determine the IgE cross-linking capability of treated and untreated samples. Moist thermal processing diminished the IgE-binding properties of both nuts, especially after heat/pressure treatment. The wheal size in SPT was importantly reduced after application of thermally-treated samples. For cashew, heat/pressure treated-samples still retain some capacity to cross-link IgE and degranulate basophils, however, this capacity was diminished when compared with untreated cashew. For pistachio, the degranulation of basophils after challenge with the harshest heat/pressure treatment was highly decreased. Boiling produced more variable results, however this treatment applied to both nuts for 60 min, led to an important decrease of basophil degranulation.

1. Introduction

Food allergy has a relevant impact on the quality of life of allergic people, and it is considered as an important lifelong persisting problem. Although the prevalence of food allergy varies depending on the geographical area, study populations analyzed and allergens studied, it is accepted that it affects up to 1–3% of the general population, reaching even 6–8% in children. Tree nuts are among the eight foods that cause the majority of allergic reactions to foods in Europe and the U.S. (Nwaru et al., 2014; Fernández Rivas, 2009). Furthermore, tree nuts are primarily responsible for fatal allergic reactions in the U.S and the U.K (Bock, Muñoz-Furlong, & Sampson, 2007; Pumphrey & Gowland, 2007). Tree nuts are included in the list of the most commonly allergenic ingredients (Regulation EU No 1169/2011/EC, OJEU 2011) and their presence in food must be indicated.

Consumption of tree nuts is on the rise due to their beneficial health effects, especially concerning risk reduction of coronary diseases and due to their rich nutritional composition. Particularly, pistachio nut contains a wide variety of healthy nutritional components, including high amounts of protein, antioxidants, minerals and low content of unhealthy fats (basically from MUFA and PUFA), among others (Bulló, Juanola-Falgarona, Hernández-Alonso, & Salas-Salvadó, 2015). Cashew nut, for its part, is highly energetic and rich in unsaturated fatty acids, fibre, amino acids and vitamins (Rico, Bulló, & Salas-Salvadó, 2015).

Typically, tree nuts allergens are identified as seed storage proteins, among others. In cashew, major allergens are characterized as a vicilin-like protein or 7S globulin (Ana o 1, 50 kDa), legumin-like protein or 11 S globulin (Ana o 2, 53 kDa) and 2S albumin (Ana o 3, 12 kDa) (Robotham et al., 2005; Wang et al., 2002; Wang, Robotham, Teuber, Sathe, & Roux, 2003). It seems that cashew allergy prevalence is increasing over the years and it has been involved in severe anaphylaxis, even exceeding peanut allergy in severity (Clark, Anagnostou, & Ewan, 2007; Van der Valk, Dubois, Gerth van Wijk, Wichers, & de Jong, 2014). Pistachio is also a well-characterized tree nut whose allergens belong to 2S albumin (Pis v 1, 7 kDa), legumin-like proteins or 11S globulins (Pis v 2 and Pis v 5, 32 and 36 kDa), vicilin-like protein or 7S globulin (Pis v 3, 55 kDa) and superoxide dismutase (Pis v 4, 25.7 kDa) (Ahn, Bardina, Grishina, Beyer, & Sampson, 2009; Noorbakhsh, Mortazavi, Sankian,

Abbreviations: BCA, Bicinchoninic acid assay; DBPCFC, double-blind placebo-controlled food challenge; FEIA, Fluor-enzyme-immunoassay; HRP, horseradish peroxidase; MRA, mediator release assay; PVDF, polyvinylidene difluoride; SPT, Skin Prick Testing; TBS, Tris buffered saline; TBST, TBS plus 0.5% Tween-20; TMB, tetramethylbenzidine

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Shahidi, & Assarehzadegan et al., 2010; Willison et al., 2008). Cross-reactivity between cashew, pistachio and mango, all of them members of *Anacardiaceae* family, has been observed (García & Lizaso, 2011; Noorbakhsh et al., 2011). Currently, there is no treatment for cashew and pistachio allergy. Therefore, avoidance is the only effective "therapy" for allergic patients. However, cashew and pistachio presence as traces is sometimes difficult to eliminate, due to cross-contamination in food lines (Taylor & Baumert, 2010).

Thermal (moist heating, dry heating, dielectric heating) and non-thermal (mechanical, enzymatic, irradiation) treatments are mainly carried out in industry to improve food quality, preservation or safety. Moreover, certain thermal processing methods are also used by consumers in order to improve sensorial properties of foods. Food processing can modify the structure and function of food proteins and may alter (by increasing or decreasing) their allergenic properties (Cabanillas & Novak, 2017). In that sense, understanding the potential effects of food processing on the allergenic properties of foods constitutes an active area of research.

In the specific case of nuts, the influence on allergenicity after a wide variety of different treatments has been studied (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015; Vanga & Raghavan, 2016; Verhoeckx et al., 2015). Thermal treatments in walnut (Cabanillas et al., 2014), HHP in hazelnut (Prieto et al., 2014), roasting, blanching, autoclaving and microwave heating in almond (Venkatachalam, Teuber, Roux, & Sathe, 2002) and several thermal processing conditions in peanut (Cabanillas et al., 2012, 2015; Maleki, Chung, Champagne, & Raufman, 2000) have been studied with different results, depending on the conditions of the treatments and the material analyzed. Knowledge about the effects of thermal processing on tree nuts such as cashew or pistachio is scarce and based on traditional *in vitro* immunoassays. Only a few studies have analyzed the influence of various treatments including autoclaving (at 121 °C), blanching, pH variation, microwave heating and γ-irradiation over cashew seeds. Although cashew proteins showed high stability to all processing methods used, autoclaving or a combination of γ-irradiation plus autoclaving seemed to cause some decrease in antigen detection (Su, Venkatachalam, Teuber, Roux, & Sathe, 2004; Venkatachalam et al., 2008). Mattison et al. (2014) found that sodium sulphite and heating treatment can modify the structure of specific cashew allergens, decreasing their IgE-binding (Mattison et al., 2014). Interestingly, the same authors also demonstrated by SDS-PAGE and LC-MS/MS that solubility of cashew proteins is modified by heat treatment and the relative amount of peptides from specific cashew allergens was also affected as well as IgE-binding capability of the soluble extracts (Mattison et al., 2016). Oleic acid has been found to bind cashew allergens, reducing the IgE-binding capacity (Chung, Mattison, Reed, Wasserman, & Desormeaux, 2015). In pistachio nuts, a reduced reactivity was observed by western blot and ELISA analysis after soaking in lemon water and steaming, without changes in sensory evaluation (Noorbakhsh, Mortazavi, Sankian, Shahidi, & Maleki et al., 2010).

An altered ability of food allergens to bind IgE using traditional *in vitro* immunoassays is not always directly related to a modified allergenic function (Shi et al., 2013). Therefore, physiologically relevant experiments, such as SPT and mediator release assays (MRA), in which the IgE cross-linking capacity of processed food proteins is analyzed in effector cells of allergy, should constitute an essential part on the research of allergenic properties of processed food. This kind of studies are important preliminary tests to ensure a possible reduction in IgE cross-linking capacity, before performing further clinical studies.

The aim of this work was to elucidate the influence of moist thermal treatments (boiling and heat/pressure) on the IgE-reactivity of cashew and pistachio proteins, by means of traditional *in vitro* immunoassays, and physiological relevant assays as SPT and MRA.

2. Materials and methods

2.1. Plant material, thermal processing and protein extraction

Cashew (*Anacardium occidentale,* type 320) obtained from Productos Manzanares S.L. (Spain) and raw pistachio (*Pistacia vera*, Kerman) from the Germoplasm Bank of Institut de Recerca i Tecnología Agroalimentàries (IRTA-Mas de Bover, Tarragona, Spain) were used for this study. Cashew nuts were not purchased as raw, since they were industrially processed in order to remove harmful oils, shell and the skin.

Nuts were boiled in distilled water (1:5 w/v) for 30 and 60 min (named as "boiled 30′" and "boiled 60′" respectively), or subjected to heat and pressure treatments in distilled water (1:5 w/v) using a Compact 40 Benchtop autoclave (Priorclave, London, UK) at 121 °C (1.18 atm) for 15 min and 30 min (named as "AU 121 °C 15′" and "AU 121 °C 30′") or 138 °C (2.56 atm) for 15 and 30 min (named as "AU 138 °C 15′" and "AU 138 °C 30′").

Untreated and treated nuts were freeze-dried (Telstar Cryodos frezze-drier), ground using a kitchen robot (Thermomix 31-1, Vorwerk Elektrowerke, GmbH & Co. KG, Wüppertal, Germany), defatted with *n*-hexane (34 ml/g of flour) and milled with a sieve of 1 mm (Tecator, Cyclotec 1093, Höganäs, Sweden). The nitrogen contents of the pistachio and cashew flours were determined by LECO analysis, according to standard procedures based on Dumas method. The total protein content was calculated as N × 5.3 (AOAC, 2000).

Proteins from treated and untreated defatted flours from cashew were extracted in a solution of Borate Buffer Saline (BSB) 1:10 w/v (100 mM H_3BO_3, 25 mM $Na_2B_4O_7$ and 75 mM of NaCl, pH 8.4), overnight at 4 °C with constant shaking. After sonication (three times 15 s), centrifugation was carried out at 8250*g* (8500 rpm) at 4 °C for 20 min. Supernatant was collected and sterilized with 0.22 μm filters. The same buffer but adding 1% polyvinylpyrrolindone (PVP) was employed to obtain pistachio protein extract from untreated and treated defatted flours at 1:10 w/v, for 1 h at 4 °C and constant stirring. After centrifugation (27419*g* or 15000 rpm, 20 min at 4 °C), supernatants were dialyzed against distilled water using a membrane with a cut-off point of 3.5 kDa for 24 h at 4 °C, and then they were freeze-dried. Pistachio dry extracts were then resuspended in sterile PBS buffer and sterilized with 0.22 μm filters. The bicinchoninic acid assay (BCA) (Pierce Biotechnology, Rockford, IL, USA) was used for protein extracts quantitation.

2.2. Patients and sera

Sera from six patients with clinical allergy to pistachio and/or cashew, confirmed on the basis of either a convincing history of anaphylaxis with positive SPT and specific serum IgE levels to pistachio and/or cashew measured by means of fluorescent enzyme immunoassay (CAP-FEIA system, Phadia, Uppsala, Sweden), shown in Table 1, or a positive double-blind placebo-controlled food challenge (DBPCFC). The study was approved by the Ethics Committee of the Hospital Universitario 12 de Octubre, Madrid, Spain (Permission No. 0312150129).

Cashew and/or pistachio allergic patients underwent SPT with untreated and treated samples according to standard methods (Malling, 1993). The mean diameters of SPT reactions were expressed in millimetres, and calculated as the sum of the largest diameter and the perpendicular distance, divided by two. SPT was performed in duplicate and a positive (histamine dihydrochloride) and negative (PBS) control were applied. Positive results were considered when wheal size was at least 3 mm greater than that elicited by the negative control. Paired *t*-test was used for comparison of means from untreated with treated samples, and differences were considered significant with $p < .05$. The statistics software GraphPad Prism version 5 for Windows (GraphPAd Software, San Diego, CA, USA) was used.

Table 1
Immunological and clinical characteristics of the 6 patients allergic to pistachio and/or cashew included in this study.

Patient	Age/Sex	Allergen	IgE (kU/L)	Symptoms	Diagnostic challenge
#1	22/F	Pistachio	2.09	OAS	DBPCFC
		Cashew	1.38	Angioedema, urticaria, cough	a
#2	46/F	Pistachio	0.69	OAS, abdominal pain	DBPCFC
		Cashew	0.81	Urticaria, abdominal pain, vomiting	a
#3	42/F	Pistachio	1.05	OAS	DBPCFC
#4	23/M	Pistachio	12.1	Urticaria, Angioedema, cough.	a
		Cashew	8.75	Urticaria, cough, dizziness	a
#5	50/F	Pistachio	0.35	Urticaria, bronchospasm	a
#6	29/M	Pistachio	21.3	OAS, angioedema	DBPCFC
		Cashew	8.94	OAS, angioedema	DBPCFC

DBPCFC, double-blind, placebo-controlled food challenge; F, female; M, male; OAS, oral allergy syndrome. a, Not challenged because of a convincing history of anaphylaxis to cashew or pistachio.

2.3. Protein electrophoresis and IgE-western blot analysis

Cashew and pistachio proteins were separated by SDS-PAGE. Twenty micrograms of protein, calculated by BCA assay, were mixed with Laemmli sample buffer and β-mercaptoethanol and heated for 10 min at 95 °C and electrophoresed in a 12% SDS-polyacrylamide gels, employing a Mini-Protean Tetra Cell apparatus (Bio-Rad, Hercules, CA, USA). Proteins were visualized with Coomassie Brilliant Blue (Bio-Rad, Hercules, CA, USA) or transferred into a polyvinylidene difluoride (PVDF) membrane (Merck KgaA, Darmstadt, Germany) for IgE-western blot analysis, using a semi-dry system (Biometra GmbH, Göttingen, Germany). Blocking was carried out for 1 h at room temperature in Tris-buffered saline containing 0.5% of Tween-20 (TBST) and 5% w/v non-fat milk. Incubation with pooled sera from the patients with pistachio (patients No. 1–6) or cashew (patients No. 1, 2, 4 and 6) allergy at 1:10 dilution was performed overnight at 4 °C. Membranes were washed and incubated with an anti-human IgE antibody produced in mouse (clone 1A2, Abbiotec, San Diego, CA USA) (stock: 1 mg/ml, used at 1:1000) for three hours at room temperature. Membranes were washed and finally treated with HRP-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) (stock: 0.4 mg/ml, used at 1:1000) for 1 h. Detection was achieved by means of enhanced chemiluminescence (SignalFire™ Elite ECL Reagent, Cell Signaling Technology Inc, Danvers, USA).

In an additional experiment, untreated and treated cashew and pistachio flours were directly solubilized in SDS sample buffer as previously described to obtain total protein (Cabanillas et al., 2014). Electrophoretic analyses of cashew and pistachio total protein extractions were carried out as previously described (Cabanillas et al., 2014). Proteins were visualized with Coomassie Brilliant Blue or transferred onto PVDF membranes for IgE-western blot analysis as explained above.

2.4. IgE-ELISA and ELISA inhibition

Polystyrene 96-well plates (BD Falcon 353279, Heilderberg, Germany) were coated with 100 µl/well of cashew and pistachio extracts from untreated or treated samples (selected treatments: "boiled 60′", "AU 121 °C 30′" and "AU 138 °C 30′"), previously diluted at 50 µg/ml in PBS pH 7, and incubated overnight at 4 °C. Wells coated with blocking solution (PBST 0.1% (v/v) and 3% (w/v) of non-fat milk) instead of protein extracts were used as negative control. After washing with PBS-Tween 20 (PBST) at 0.5% (v/v), wells were blocked with blocking solution, for 1 h at room temperature. Plates were incubated with pooled sera or individual sera from the patients with pistachio and/or cashew allergy at 1:10 for 2 h at 37 °C, washed and treated with mouse anti-human IgE antibody (clone 1A2, Abbiotec, San Diego, CA USA, stock 1 mg/ml, used at 1:1000 dilution in blocking solution) for 1 h at 37 °C. After washing the wells, HRP-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Dallas, Texas, USA; Stock at 0.4 mg/ml, used at 1:1000 dilution) was added and incubated for 1 h at 37 °C. The reaction was developed with tetramethylbenzidine (TMB) and H_2O_2 substrate (R & D Systems, Minneapolis, USA), stopped with sulfuric acid 1M and OD was measured at 450 nm with 650 nm as a reference. All the tests were performed in duplicate. Cut-off point of positivity was calculated with the formula: mean OD + 3 × SD for the negative control, as described in previous studies (Palacin et al., 2007; Cabanillas et al., 2015); and it was represented as a horizontal line in the graphics. For the analysis of the results of ELISA performed with individual sera, paired *t*-test was used. Differences were considered as significant with $p < 0.05$. The statistics software GraphPad Prism version 5 for Windows was used.

For the ELISA inhibition experiment, polystyrene 96-well plates (BD Falcon 353279, Heidelberg, Germany) were coated with 100 µl/well of untreated pistachio or cashew at a final concentration of 250 µg/ml and incubated overnight at 4 °C. At the same time, in parallel, a pooled sera of pistachio or cashew allergic patients (final dilution 1:10) were pre-incubated with untreated or treated pistachio or cashew protein extracts as inhibitors (selected treatments: "boiled 60′", "AU 121 °C 30′" and "AU 138 °C 30′") at different final concentrations: 1, 0.1, 0.01 and 0.001 mg/ml, overnight at 4 °C and soft stirring. Pooled sera pre-incubated with PBS were also included (non-inhibited serum). Wells were washed and blocked with PBST 3% non-fat milk for 1 h. Incubation of the wells with the sera pre-incubated with the different inhibitors or the non-inhibited serum was carried out for 3 h at 37 °C. After washing the wells, incubations with mouse anti-human IgE antibody at 1:1000 for 1 h at 37 °C and HRP-conjugated goat anti-mouse IgG antibody at 1:1000 dilution were performed, and OD was measured at 450 nm with 650 nm as a reference. The percentage of inhibition was calculated with the formula: $[1 - (A_I/A_N)] \times 100$, where A_I is the absorbance value obtained in the wells incubated with inhibited serum and A_N the absorbance of the wells incubated with the non-inhibited serum (Cabanillas et al., 2015).

2.5. Rat basophil leukemia cell line (RBL-48) for MRA

RBL-48 cell line, transfected with the α chain from the high-affinity human IgE receptor FcεRI (a gift from Dr. J. Kochan) (Gilfillan et al., 1992), was used in order to analyze the release of allergic mediator β-hexosaminidase, induced by untreated and treated cashew and pistachio protein extracts. Cells were cultured in very low endotoxin RPMI 1640 Medium (Sigma-Aldrich, Saint Louis, MO, USA), supplemented with 10% heat-inactivated fetal calf serum, 1% antibiotic/antimycotics and 500 µg/ml of geneticin. The expression of the FcεRI-α chain was confirmed by flow cytometry, using an anti-human FcεRIα subunit antibody (eBioscience Inc, San Diego, CA, USA). Fifty µl of cells at 1×10^6/ml were plated in a 96-well tissue culture plate (Corning Inc, NY, USA) and sensitized with the pooled sera from allergic patients to cashew (patients No. 1, 2, 4 and 6) or pistachio (patients No. 1–6) at 1:10 final dilution, overnight at 37 °C, 5% of CO_2 and 95% humidity. Cells were washed with Tyrodes buffer and then stimulated for 1 h with sterile untreated and treated cashew or pistachio protein extracts at 1 mg/ml (selected treatments: "boiled 60′", "AU 121 °C 30′" and "AU 138 °C 30′"). Cells stimulated with Tyrodes Buffer instead protein extracts were used as a negative control. This negative control provides a measurement of the spontaneous release of mediator to the media alone. RBL-48 cells degranulation was measured by β-hexosaminidase release as previously described (Cabanillas et al., 2014). RBL-48 cells were lysed with 1% Triton X-100 for total mediator release. Percentage

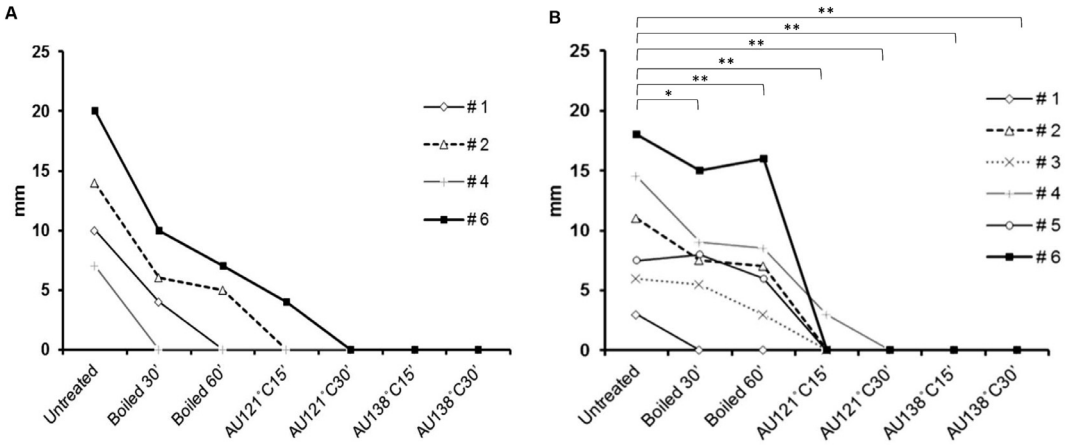

Fig. 1. SPT in patients with cashew and pistachio allergy. SPT with untreated and treated samples of cashew (A) and pistachio (B) in 4 and 6 patients with clinical allergy to cashew and pistachio, respectively. The mean diameters of the wheals in mm are shown. Significant differences with $^{*}p < .05$; $^{**}p < .005$ determined using paired *t*-test for pistachio.

of β-hexosaminidase release was calculated as previously described (Cabanillas et al., 2014). Assays were performed in triplicate.

3. Results

3.1. SPT

SPT were carried out in cashew and pistachio allergic patients to determine the IgE cross-linking capability of untreated and all treated samples (boiled 30 and 60 min, heat/pressure 121 °C, 1.18 atm, 15 and 30 min and heat/pressure 138 °C, 2.56 atm, 15 and 30 min). Data are represented in Fig. 1. All patients had positive SPT with untreated cashew and pistachio protein extracts, and none of the patients had a positive result with AU 121 °C 30′, AU 138 °C 15′ and AU 138 °C 30′ extracts in both nuts. In pistachio, in which the statistical analysis using paired *t*-test was possible, a statistically significant decrease in the allergenic potential compared to untreated extracts was found for the mentioned treated samples. A decrease in the wheal size with boiled cashew and pistachio compared with the untreated samples was also found, although the decrease was not as strong as the one produced with heat-pressure samples. Even so, the decreased in the wheal size with boiled pistachio was significant, especially for the sample boiled for 60 min.

3.2. Immunodetection assays of processed cashew

3.2.1. Electrophoretic pattern and IgE-western blot of cashew samples

The characterization of the electrophoretic profile of the soluble protein extracts from untreated and thermal-treated cashew samples is shown in Fig. 2A. IgE-binding proteins were analyzed by IgE-western blot, using pooled sera from the 4 cashew allergic patients (Fig. 2B). The results showed that the treatments of boiling during 30 min had no major effects in the SDS-PAGE profile and the IgE-binding proteins from cashew. High molecular weight proteins (around 50 kDa) were the first bands affected by treatments (boiling 60 min) in SDS-PAGE and IgE-western blot. Cashew subjected to heat and pressure treatments showed less distinctive stained bands in SDS-PAGE with an increased protein fragmentation that went along with a reduction in IgE-reactive bands (Fig. 2B).

We additionally studied the electrophoretic and IgE-binding patterns of total protein from cashew which were obtained by direct solubilization of untreated and treated cashew flours in SDS sample buffer as described in materials and methods. The results showed that the electrophoretic and IgE-binding pattern profiles of total protein were similar to soluble protein extract (Supplementary material Fig. 1), although a band below 15 kDa was especially immunoreactive as well as resistant to applied processing. The IgE reactive bands were strongly reduced in the samples treated with heat and pressure, but still detectable even in the sample AU 138 °C 15 min.

3.2.2. IgE-ELISA and ELISA inhibition with cashew samples

Untreated and the selected treated cashew samples: boiled 60 min, AU 121 °C 30 min and AU 138 °C 30 min were used for these experiments.

ELISA inhibition assay was carried out using the pooled sera from the 4 patients with cashew allergy. Untreated cashew proteins were used to coat the plate and the three thermally treated samples (boiled 60 min, AU 121 °C 30 min and 138 °C 30 min) and untreated sample (control) were used as inhibitors mixed with the pooled sera at different concentrations. Results showed that proteins from untreated and boiled samples at 1 mg/ml inhibited 94% of the IgE-binding to untreated cashew proteins coated in the wells. The results also showed a decrease in IgE-binding capacity for heat/pressure treated proteins, showing a percentage of inhibition which became stagnant for AU 121 °C 30′ treatment at around 45% at 0.01, 0.1, and 1 mg/ml. For the treatment AU 138 °C 30′ the inhibition was about 0% up to 0.01 mg/ml and maximum of 37% at the highest concentration of inhibitor tested (Fig. 2C), which indicates a marked decrease in IgE-binding capacity for this specific treated cashew sample.

These results were confirmed by IgE-ELISA, in which the wells were coated with untreated and selected treated proteins and the pooled sera from cashew allergic patients were used. Results showed around 90% of reduction of IgE reactivity in heat/pressure treated samples and no marked effect was obtained after boiling treatment. The reduction of IgE reactivity after thermal treatments of cashew nuts was also analyzed by means of IgE-ELISA with the individual sera from the four patients with clinical allergy to cashew (1, 2, 4 and 6). IgE reactivity was strongly reduced after heat/pressure treatments (AU 121 °C 30 min and AU 138 °C 30 min). Boiling for 60 min, however, reduced IgE reactivity at a lesser extent in IgE-ELISA.

3.3. Immunodetection assays of processed pistachio

3.3.1. Electrophoretic pattern and IgE-western blot of pistachio samples

The protein profile, visualized by SDS-PAGE, of pistachio protein extract from untreated and boiling treated samples was very similar, and only a few high molecular weight bands were degraded. Moreover, some bands, mainly above 35 kDa, were reduced after the softest heat/pressure treatment (AU 121 °C, 15 min). The rest of heat/pressure treatments, especially AU 138 °C, 2.56 atm (15 min and 30 min) provoked a smear due to the degradation, rich in low molecular weight proteins. The strongest processing effect was obtained after harsh heat/

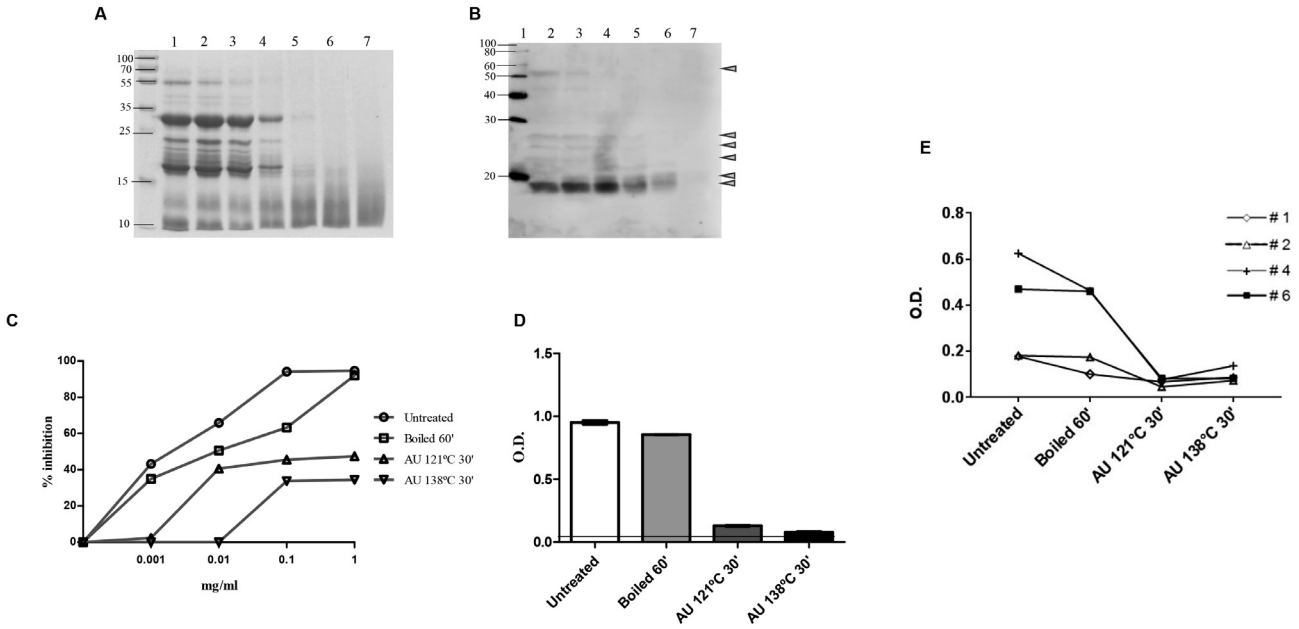

Fig. 2. Protein profile and IgE-immunodetection of cashew extracts. (A) SDS-PAGE and (B) IgE-western blot of cashew protein extract from untreated (lane 1) and treated nuts: boiled 30′ (lane 2), boiled 60′ (lane 3), AU 121 °C, 15′ (lane 4), AU 121 °C, 30′ (lane 5), AU 138 °C, 15′ (lane 6) and AU 138 °C, 30′ (lane 7). IgE-western blot was performed using pooled sera from four patients allergic to cashew (patients 1, 2, 4, and 6). IgE-reactive bands are marked with arrows. C. ELISA inhibition assay with untreated cashew extract for coating and untreated and treated cashew proteins (indicated in the legend) used as inhibitors. Pooled sera from four patients allergic to cashew was used (patients 1, 2, 4, and 6). D. IgE-ELISA of untreated and treated cashew incubated with the pooled sera. The cut-off point of positivity is indicated with a horizontal line. E. IgE-ELISA of untreated and treated cashew incubated with individual sera.

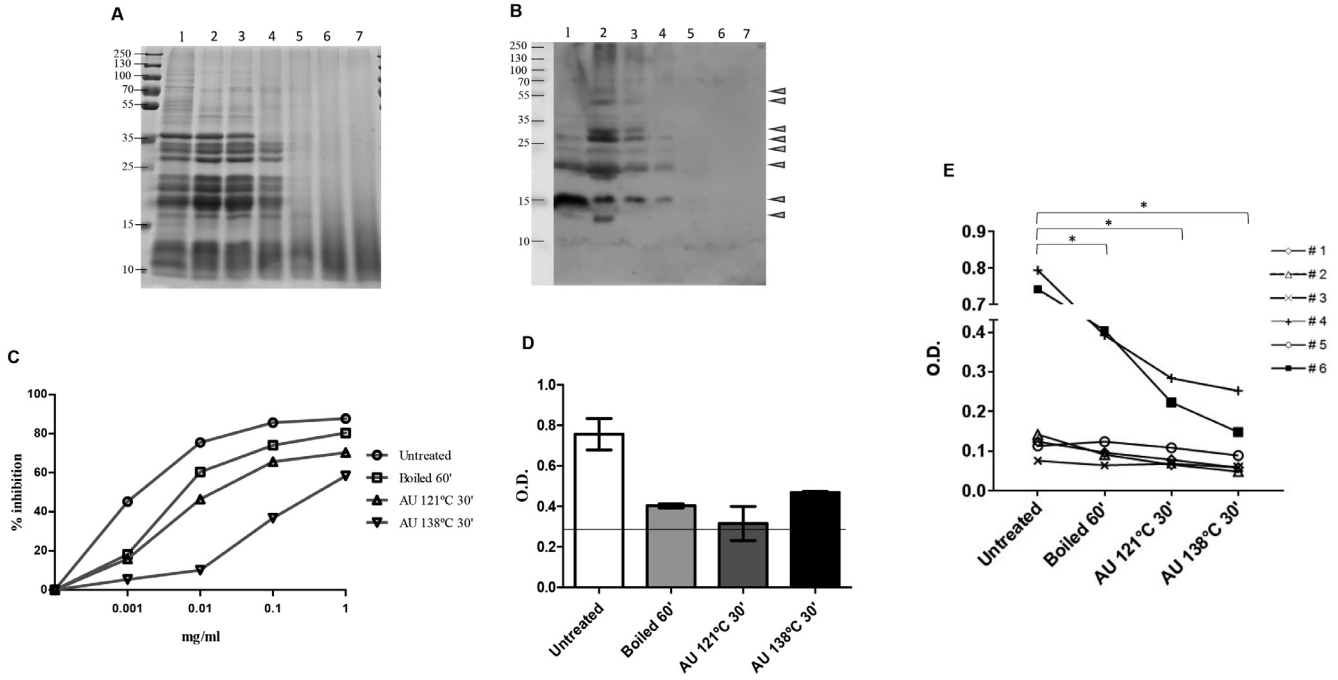

Fig. 3. Protein profile and IgE-immunodetection of pistachio extracts. (A) SDS-PAGE and (B) IgE-western blot of pistachio protein extract from untreated (lane 1) and treated nuts: boiled 30′ (lane 2), boiled 60′ (lane 3), AU 121 °C, 15′ (lane 4), AU 121 °C, 30′ (lane 5), AU 138 °C, 15′ (lane 6) and AU 138 °C, 30′ (lane 7). IgE-western blot was performed using pooled sera from six patients allergic to pistachio (patients 1–6). IgE-reactive bands are marked with arrows. C. ELISA inhibition assay with untreated pistachio extract for coating and untreated and treated pistachio proteins (indicated in the legend) used as inhibitors. A pooled sera from six patients allergic to pistachio was used. D. IgE-ELISA of untreated and treated pistachio incubated with pooled sera. The cut-off point of positivity is indicated with a horizontal line. E. IgE-ELISA of untreated and treated pistachio incubated with individual sera. Significant differences are determined with $^{*}p < .05$ using the paired *t*-test.

pressure conditions (138 °C for 30 min) (Fig. 3A). The protein migration of pistachio flour directly solubilized in the electrophoretic sample buffer was also analyzed and no relevant differences compared to pistachio soluble protein extract were detected (Supplementary material Fig. 1).

IgE-western blot was performed with total and soluble protein extraction, using a pool of six patients' sera with pistachio allergy (1 −6). IgE-reactivity was detected for several bands up to heat/pressure treatment at 121 °C for 15 min. Two bands were especially resistant (bands around 20 and 13 kDa), but also bands in the range from 30 to

55 kDa were easily observable in soluble protein extract western blot (Fig. 3B). The band around 13 kDa was detected in the IgE-western blot of pistachio total protein, even at 121 °C 30 min (Supplementary material Fig. 1). The soluble protein extracts from untreated and the selected treated samples: boiled 60′, AU 121 °C 30′ and AU 138° C 30′ were used for the rest of experiments.

3.3.2. IgE-ELISA and ELISA inhibition with pistachio samples

Inhibition of IgE-binding to immobilized untreated pistachio proteins augmented with increased concentrations of thermal-treated pistachio extracts (inhibitors). Untreated and boiled 60′ samples competed for IgE at 87% and 80% at 1 mg/ml respectively. Pistachio treated with heat/pressure at 138 °C for 30 min was a weaker competitor than boiled or soft heat/pressure treatment at all concentrations, reaching a maximum of 58% of inhibition at 1 mg/ml, which indicates that is the treatment that caused the major decrease in IgE-binding capacity (Fig. 3C).

A consistent IgE-reactivity decrease after boiling and heat/pressure treatments was observed in IgE-ELISA test with a pooled sera from the 6 patients allergic to pistachio (Fig. 3D). The results obtained with IgE-ELISA using individual sera from the six allergic patients (1–6) showed a significant decrease in IgE-reactivity for the three treatments compared with untreated pistachio (Fig. 3E).

3.4. MRA to assess thermal effect on cashew and pistachio allergens

The differential IgE cross-linking capability of untreated or treated cashew and pistachio allergens was analyzed by β-hexosaminidase release assay using the RBL-48 cell line. The cell line showed a consistent expression of the FcεRI-α chain (more than 90% of the cell population), analyzed by flow cytometry before sensitization and mediator release assays (Fig. 4A). The cultured RBL-48 cell line was sensitized with a pooled sera from cashew or pistachio allergic patients, and afterwards, cells were stimulated with untreated or treated (boiled 60′, AU 121 °C, 30′, and AU 138 °C, 30′) cashew or pistachio protein extracts.

Results showed that untreated cashew and pistachio provoked a β-hexosaminidase release of 9 and 21%, respectively (Fig. 4B and C). In both cases, boiling for 60 min and heat/pressure processing showed a relevant lower capacity to trigger degranulation of RBL-48 cells, reducing the percentage of mediator release around 60% compared to the untreated samples. In pistachio, the degranulation of RBL-48 cells after the challenge with the harshest heat/pressure treatment (AU 138 °C 30′) was highly decreased, effect that was more attenuated in cashew treated with the same thermal conditions. Heat/pressure treated-cashew seemed to be able to cross-link IgE on basophils and to induce the β-hexosaminidase release to the media, although this capacity was diminished when compared with untreated cashew.

4. Discussion

In this study, the influence of moist thermal treatments on the IgE-reactivity of cashew and pistachio has been evaluated by traditional immunoassays such as IgE-ELISA, inhibition ELISA or IgE-western blot and by *in vivo* and physiologically relevant assays, as SPT and MRA that evaluate the IgE cross-linking capacity of untreated and treated proteins on effectors cells of allergy. All the results corroborated that heat and pressure treatment at the harshest conditions considered (AU 2.56 atm, 138 °C 30 min) produced an overall decrease in IgE-binding of both tree nuts, analyzed by IgE-western blot and IgE ELISA, using pooled or individual sera. Interestingly, applied treatments of heat and pressure seemed to affect cashew allergens to a greater extent than pistachio allergens, in regard to the IgE-binding capacity (evaluated by IgE-ELISA, indirect and by inhibition). Results went along with a marked decrease in the wheal size in SPT due to heat and pressure treatments in both tree nuts. Higher sensitivity of cashew proteins to boiling processing compared to pistachio was observed by this test. SPT showed negative reactions in all patients after applying cashew and pistachio protein extracts from AU 121 °C 30 min and AU 138 °C for 15 and 30 min samples. The effects of thermal processing on the allergenic properties of cashew and pistachio have been addressed by a limited number of studies, especially in the case of pistachio, based on assays that evaluated IgE-binding in western blot or ELISA (Masthoff et al., 2013). In 2008, Venkatachalam et al., found that cashew allergens had high stability to a wide variety of treatments assayed, including pressure cooking at 121 °C (during 5, 10, 20, and 30 min), boiling (during 1, 4, 7, and 10 min), microwave heating, dry roasting, and non-thermal treatments such as γ-irradiation or pH variation. Interestingly, although high stable, cashew allergens seem to be affected at some extent only by the treatment that applied heat and pressure (autoclaving) at 121 °C for the longest period of time from the plethora of treatments analyzed. Mattison et al. (2016) applied dry heat treatment to cashew (149 °C for 12, 20 and 24 min, and 177 °C for 24 min) and found that the soluble protein profile of cashew was altered after roasting. Consequently, processing seemed to change the relative amount of specific allergenic peptides, and IgE-binding capability was reduced to a less than 60% after dark roasting at 149 °C for 24 min. There is less information about thermal effect on pistachio immunoreactivity or IgE-binding capability. Noorbakhsh et al. found lower IgE-binding capacity for the protein extract prepared from steam-roasted than from raw and dry-roasted pistachio nuts (Noorbakhsh, Mortazavi, Sankian, Shahidi, Maleki, et al.,

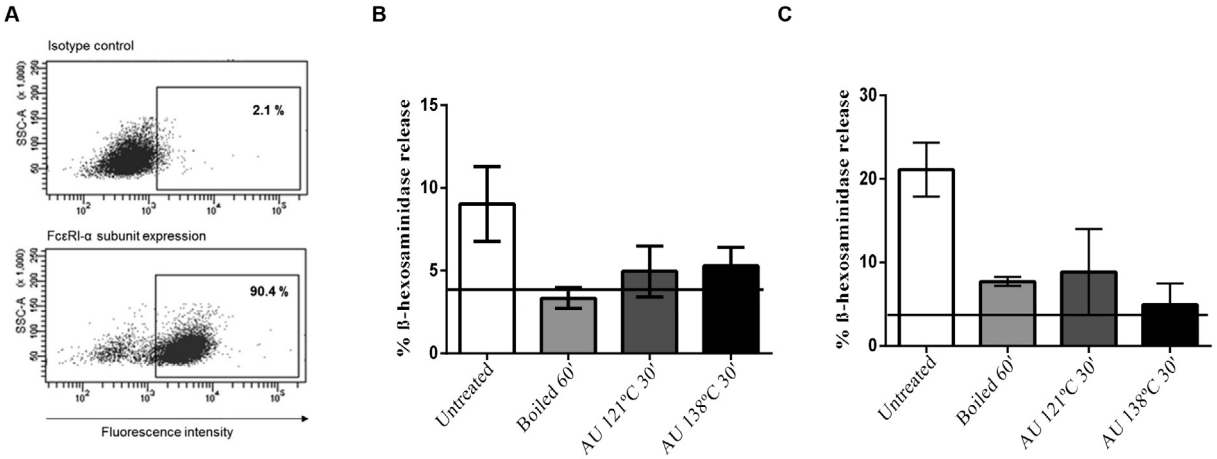

Fig. 4. Mediator release assay. Analysis of the surface expression of the human receptor FcεRI (α subunit) by means of flow cytometry on RBL-48, performed before serum sensitization and mediator release assay (A). Percentages of β-hexosaminidase release from RBL-48 sensitized with pooled sera of 4 cashew (B) or 6 pistachio (C) allergic patients. Sensitized cells were challenged with untreated and treated cashew (B) or pistachio (C). The mediator release value from negative control is indicated with a horizontal line in the graphs.

2010). In our study, we have applied thermal treatments to cashew and pistachio that included not only boiling without pressure and heat/pressure treatments at soft conditions, but also harsher conditions of heat and pressure (138 °C, 2.56 atm, during 15 and 30 min), which turned to be the most efficient treatment to decrease the allergenic properties of both tree nuts considered in our study.

IgE cross-linking capability of proteins from cashew and pistachio was also affected by all the applied treatments. However, the results of MRA using the cell line RBL-48, seemed to indicate that cashew proteins treated with heat and pressure, although importantly diminished compared to untreated cashew protein extract, still retained some capacity to cross-link IgE. This result indicates that an altered ability of food allergens to bind IgE using traditional *in vitro* immunoassays, such as IgE-western blot and IgE-ELISA for cashew, is not always correlated to an equal alteration of the IgE cross-linking capacity (Shi et al., 2013). In pistachio, however, the degranulation of RBL-48 cells after challenge with the harshest heat/pressure treatment was highly decreased, with a value similar to the negative control (less than 5% of mediator release). Recent studies have also found some discrepancies in the effect of processing (thermal, enzymatic, etc) on food allergenicity when using traditional immunoassays and assays that analyze the capacity of treated proteins to trigger the release of allergic mediators (Panda, Tetteh, Pramod, & Goodman, 2015; Shi et al., 2013). As observed with cashew in our study, the residual degranulation of effector cells obtained after the challenge with cashew proteins treated with heat and pressure may be explained by the survival of part of IgE-binding epitopes, which are able to cross-link IgE although in a less efficient way than untreated cashew extract. Other studies, however, have found a good correlation between variations on IgE-binding capacity of thermal treated nuts in IgE-ELISA or IgE-western blot and an altered capacity to cross-link IgE in MRA (Cabanillas et al., 2014, 2015).

The harshest conditions of heat and pressure applied in our study produced a degradation of cashew and pistachio proteins, with an increased protein fragmentation seen as an intense smear in the low molecular weight area in the SDS-PAGE. Such alteration in the electrophoretic and IgE-binding patterns after heat and pressure treatments cannot be explained by a potential loss of solubility of proteins due to the thermal treatments, since the experiments carried out with strong conditions of protein solubilization (flours directly solubilized in SDS-PAGE sample buffer), showed the same pattern of protein degradation for heat and pressure-treated samples. The degradation of proteins after harsh heat/pressure treatments obtained in our study is similar to the degradation produced by some enzymatic treatments. In that sense, Kulis et al. (2012), showed a drastic difference in the electrophoretic pattern after 30 min of pepsin digestion in cashew proteins, with an evident degradation of the main cashew proteins, translated into a strong increase in protein fragments around 3–6 kDa, similar to the results obtained in our study. Interestingly, the authors found that such hydrolyzed cashew sample significantly decreased the allergenicity in a mouse model of cashew allergy. Furthermore, immunotherapy with such pepsinized cashew in orally sensitized mice induced IgG production and decreased Th2 cytokine responses (Kulis et al., 2012). In our study, we have found a marked decrease in the IgE-binding properties of cashew and pistachio proteins, with a reduced capacity to cross-link IgE in effectors cells of allergy, especially in the case of pistachio. However, the potential use for immunotherapy of cashew and pistachio subjected to harsh conditions of heat and pressure will be a matter of future studies. Several studies have proposed the use of peptides with reduced IgE cross-linking capacity as an attractive strategy for immunotherapy (Novak, Haberstok, Bieber, & Allam, 2008). Recently, it has been demonstrated that peanut boiled during 12 h showed a protein fragmentation with an increased number of peptides with a diminished capacity to bind IgE, but with the ability to activate antigen-specific T cells, an essential step for successful oral immunotherapy (Tao et al., 2016). In other foods, such as milk or egg, it has been demonstrated in clinical trials that around 70% of the children with milk and egg allergies included in the study tolerated heated milk or egg products, with decreased wheal sizes in SPT and increased levels of specific IgG4 antibodies (Lemon-Mulé et al., 2008; Nowak-Wegrzyn et al., 2008).

The limitation of our study includes the use of a relative small study population of cashew and/or pistachio allergic patients. However, the patients included here were not only sensitized to cashew and/or pistachio, but also had well-characterized clinical allergies to cashew and/or pistachio.

In conclusion, the results of our study indicate that heat/pressure treatments were able to decrease the IgE-binding properties of cashew and pistachio protein extracts evaluated in IgE-ELISA and IgE-western blot. SPT and MRA assays confirmed a diminished capacity to cross-link IgE for pistachio samples. In cashew, although heat and pressure treated samples still retain some capacity to trigger the release of allergic mediators in cells implicated in the allergic response, this capacity was diminished when compared with untreated sample. Boiling produced more variable results, however this treatment applied to both nuts for 60 min led to an important decrease of basophil degranulation. Further studies will be necessary to analyze the decreased IgE cross-linking capacity of heat/pressure treated samples in *in vivo* models of food allergy. Furthermore, the potential capacity of such treated samples in the induction of T cell reactivity for a potential use in oral immunotherapy should be also addressed in future studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.10.132.

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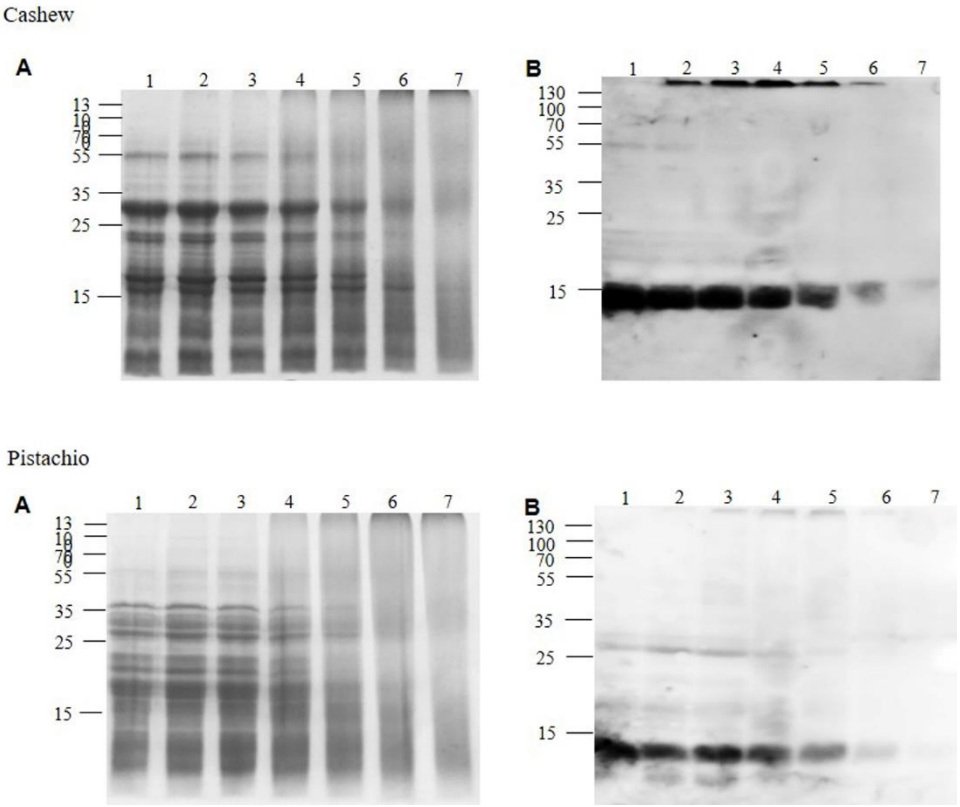
Appendix 1. Supplementary Material



Supplementary material. Figure 1. Protein profile and immunodetection of cashew and pistachio flours. (A) SDS-PAGE and (B) IgE-western blot of cashew or pistachio flours directly solubilized in electrophoresis SDS sample buffer as previously described (Cabanillas et al., 2014). Untreated (lane 1) and treated nuts: boiled 30' (lane 2), boiled 60' (lane 3), AU 121 °C, 15' (lane 4), AU 121 °C, 30' (lane 5), AU 138 °C, 15' (lane 6) and AU 138 °C, 30' (lane 7). IgE-western blots were performed using pooled sera from patients allergic to cashew or pistachio.

ARTÍCULO 2.

INFLUENCIA DE LA HIDRÓLISIS ENZIMÁTICA EN LA REACTIVIDAD ALERGÉNICA DE ANACARDO Y PISTACHO PROCESADOS.

INFLUENCE OF ENZYMATIC HYDROLYSIS ON THE ALLERGENIC REACTIVITY OF PROCESSED CASHEW AND PISTACHIO.

Las alergias a anacardo y pistacho están consideradas un serio problema para la salud. Estudios previos han mostrado que el procesado térmico, presurización e hidrólisis enzimática podría reducir las propiedades alergénicas de los alimentos, modificando la estructura proteica. Este estudio evalúa las propiedades alergénicas de anacardo y pistacho tras el tratamiento térmico (cocción), con o sin presión (autoclave) y tratamientos enzimáticos bajo sonicación, mediante SDS-PAGE, western blot y ELISA, usando sueros IgE de pacientes alérgicos, y espectrometría de masas. El tratamiento de autoclave y la hidrólisis enzimática con sonicación inducen una reducción cuantitativa en las propiedades de unión a IgE de pastas de harinas de anacardo y pistacho procesados. Estos tratamientos fueron más efectivos con los alérgenos de pistacho. Sin embargo, en el caso de anacardo, fue necesario combinar calor con digestión enzimática para obtener una reducción notable de su capacidad de unión a IgE. Se identifican las condiciones de procesado simultáneo más efectivas para reducir e incluso abolir el potencial alergénico de anacardo y pistacho.

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Influence of enzymatic hydrolysis on the allergenic reactivity of processed cashew and pistachio

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ABSTRACT

Cashew and pistachio allergies are considered a serious health problem. Previous studies have shown that thermal processing, pressurization and enzymatic hydrolysis may reduce the allergenic properties of food by changing the protein structure. This study assesses the allergenic properties of cashew and pistachio after thermal treatment (boiling and autoclaving), with or without pressure (autoclaving), and multiple enzymatic treatments under sonication, by SDS-PAGE, western blot and ELISA, with serum IgE of allergic individuals, and mass spectroscopy. Autoclaving and enzymatic hydrolysis under sonication separately induced a measurable reduction in the IgE binding properties of pastes made from treated cashew and pistachio nuts. These treatments were more effective with pistachio allergens. However, heat combined with enzymatic digestion was necessary to markedly lower IgE binding to cashew allergens. The findings identify highly effective simultaneous processing conditions to reduce or even abolish the allergenic potency of cashew and pistachio.

1. Introduction

Food allergy affects 6–8% of children and approximately 2% of the population. It has been estimated that food allergy causes approximately 30,000 anaphylactic reactions and 2000 hospitalizations annually in the U.S. (Jerschow, Lin, Scaperotti, & McGinn, 2014). This increase in the prevalence and severity of food allergy has led to growing concerns by consumers and the food industry (Taylor & Hefle, 2001). Peanuts and tree nuts are the leading cause of fatal anaphylactic reactions caused by foods (Bock, Munoz-Furlong, & Sampson, 2001) and have a significant impact on the quality of life (Primeau et al., 2000). The prevalence of allergy to nuts is estimated to be approximately1% in the UK and in the U.S. (Sicherer, Munoz-Furlong, Godbold, & Sampson, 2012). In Spain nuts are ranked second among foods most frequently involved in anaphylactic reactions (Fernandez-Rivas, 2009). Most nut allergens are seed storage proteins such as vicilins (7S globulin subunits composed of approximately 50–60 kDa), legumins (11–13S globulins composed of acidic subunits of 30–40 kDa and basic subunits of 15–20 kDa) and 2S albumins (15–20 kDa) (Crespo, James, Fernandez-Rodriguez, & Rodriguez, 2006). Other allergens of nuts with known biological function, such as lipid transfer proteins (LTP), profilins and pathogenesis-related, class 10 (PR-10) proteins are considered panallergens because they contribute to the allergenicity of a large group of pollen, nuts, seeds, fruits and other plants (Radauer & Breiteneder, 2007). A common feature of the nut allergenic proteins is their resistance to proteolysis and denaturation (Roux, Teuber, & Sathe, 2003).

Cashew (*Anacardium occidentale*) allergy is thought to be the second most allergic nut in the U.S. and may cause more severe reactions than peanuts (Clark, Anagnostou, & Ewan, 2007). Three allergenic proteins of cashew have been identified and characterized: Ana o 1 (7S vicilin, 50 kDa) (Wang et al., 2002), Ana o 2 (11S legumin, 55 kDa) (Wang, Robotham, Teuber, Sathe, & Roux, 2003) and Ana o 3 (2S albumin, 14 kDa) (Robothan et al., 2005). Pistachio (*Pistacia vera*) is another well characterized nut for its allergenic potential and cross-reactivity with cashew and mango (Noorbakhsh et al., 2011). Similar to other nuts, 4 of the five pistachio major allergens identified correspond to seed storage proteins: Pis v 1 (2S albumin, 7 kDa), Pis v 2 (11S legumin, 32 kDa), Pis

Abbreviations: HRP, Horseradish peroxidase; RT, room temperature

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Table 1
The description of the processing treatments and protein content, determined by LECO analysis, for cashew and pistachio samples of this study.

Code	Sample	Treatment	Protein content (g/100gr)
PDF 1	Defatted Flour Pistachio	Untreated (Control)	34.34
PDF 2	Defatted Flour Pistachio	Boiled 30 min	35.51
PDF 3	Defatted Flour Pistachio	Boiled 60 min	37.37
PDF 4	Defatted Flour Pistachio	Autoclaved 120 kPa, 15 min	41.08
PDF 5	Defatted Flour Pistachio	Autoclaved 120 kPa, 30 min	41.92
PDF 6	Defatted Flour Pistachio	Autoclaved 256 kPa, 15 min	43.20
PDF 7	Defatted Flour Pistachio	Autoclaved 256 kPa, 30 min	43.35
CDF 1	Defatted Flour Cashew	Untreated (Control)	33.06
CDF 2	Defatted Flour Cashew	Boiled 30 min	40.17
CDF 3	Defatted Flour Cashew	Boiled 60 min	37.71
CDF 4	Defatted Flour Cashew	Autoclaved 120 kPa, 15 min	36.63
CDF 5	Defatted Flour Cashew	Autoclaved 120 kPa, 30 min	35.41
CDF 6	Defatted Flour Cashew	Autoclaved 256 kPa, 15 min	42.90
CDF 7	Defatted Flour Cashew	Autoclaved 256 kPa, 30 min	37.81

v 3 (7S vicilin, 55 kDa) and Pis v 5 (11S legumin, 36 kDa) and a superoxide dismutase, Pis v 4 (25.7 kDa) (Ahn, Bardina, Grishina, Beyer, & Sampson, 2009; Ayuso, Grishina, & Ahn, 2007; Willison et al., 2008).

Food processing, particularly heat treatment, may alter the allergenicity of foods, and can therefore be useful in controlling allergenic risk (Besler, Steinhart, & Paschke, 2001; Maleki, 2004). Thermal treatment changes the structure, function, digestibility, solubility and immunogenicity of proteins, and therefore the overall allergenicity of the food (Cabanillas, Pedrosa et al., 2012; Liu et al., 2010; Maleki, 2004; Maleki & Hurlburt, 2004; Maleki, Schmitt, Galeano, & Hurlburt, 2014; Mueller, Maleki, & Pedersen, 2014; Nesbit, Hurlburt, Schein, Cheng, & Maleki, 2012; Noorbakhsh et al., 2010; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010a). These effects depend on the temperature, type and duration of the treatment, the intrinsic characteristics of the protein and the physicochemical conditions of the microenvironment (Davis, Smales, & James, 2001; Maleki, Chung, Champagne, & Raufman, 2000; Nesbit et al., 2012; Schmitt et al., 2010; Wal, 2003). There are no general rules about the effect of processing on allergenicity. Processing can lead to the generation of new allergenic epitopes (neoallergens), as well as abolish the existing epitopes (Cuadrado et al., 2009; Maleki et al., 2000; Nesbit et al., 2012; Álvarez-Álvarez et al., 2005). Several studies have evaluated the changes induced by roasting, boiling, microwave heating, and pressure-cooking on legume and nut allergenicity, showing that processing based on pressure and certain heat treatments seems to have an important impact on in vitro IgE binding capacity (Cabanillas, Maleki et al., 2012; Cuadrado et al., 2009, 2011; Maleki et al., 2000; Schmitt et al., 2010; Álvarez-Álvarez et al., 2005). A study conducted with raw, dry roasted and steamed pistachio showed that immunoreactivity decreases only when roasting is performed with steam (Noorbakhsh et al., 2010). More recently, it has been shown that the multi-enzyme systems that combine the action of endo and exopeptidases cause significant destruction of IgE epitopes in peanut and lentil (Cabanillas, Pedrosa et al., 2012; Cabanillas et al., 2010). Cabanillas et al. (2014) also found that walnuts subjected to high pressure were more susceptible to gastric and duodenal digestion.

The main objective of this study is to assess the digestibility and human serum IgE binding capacity of both soluble and insoluble pistachio and cashew proteins after thermal treatment in combination with high pressure (autoclaving), enzymatic digestion and ultrasound treatments.

2. Materials and methods

2.1. Patients and sera

Sera from 7 patients (P1-P7) with pistachio and cashew allergy, confirmed on the basis of either a convincing history or recently documented reaction after nut ingestion, were used in this study (see Table 1 suppl). The study was approved by the Ethics Committee of Tulane Health Science Center (New Orleans, LA, USA) in accordance with the rules and regulations of the institutional review board.

2.2. Plant material and processing

Cashew (*Anacardium occidentale,* type 320) obtained from Productos Manzanares (Spain) and pistachio (*Pistacia vera*, variety Kerman) from the Germoplasm Bank of Institut de Recerca i Tecnología Agroalimentàries (IRTA-Mas de Bover, Tarragona, Spain) were used in the study. Whole nuts seeds were immersed in distilled water (1:5 w/v) and boiled (100 °C, 30 and 60 min) or autoclaved using an autoclave Compact 40 Benchtop (Priorclave, London, UK) at 121 °C, 120 kPa, for 15 and 30 min and at 138 °C, 256 kPa, for 15 and 30 min. Untreated, boiled and autoclaved nut seeds were ground and defatted with n-hexane (34 ml/g of flour) for 4 h, shaken, and air-dried after filtration of the n-hexane. Defatted flour from untreated cashew and pistachio were the controls for boiled and autoclaved samples. The nitrogen contents of the samples were determined by LECO analysis according to standard procedures based on Dumas method (AOAC, 2003). The total protein content was calculated as N × 5.3 (AOAC, 2003). The analyses were carried out in duplicate and the results summarized in Table 1. The protein content of processed samples was higher than in the raw ones. This could be related to the reduction of dry matter (i.e. carbohydrates) in these processed samples.

Seven food-grade proteases (Amano Enzyme Europe Ltd., Agno, Switzerland) were tested for digestion of cashew and pistachio samples and are referred to as E1-E7 in this study: E1 – Themoase PC10F (endoprotease), E2 – ProteAX (exoprotease), E3 – Protin SD – NY10 (proprietary), E4 – Peptidase R (exopeptidase), E5 – Protin SD – AY10 (alkalase-like), E6 – Protease M “Amano” SD (proprietary), E7 – Protease P “Amano” 3SD (proprietary). All are stable at 50–55 °C and pH 7. The first three were recommended by Amano for the potential to digest allergenic proteins (E1-E3). Digestion of cashew and pistachio protein extracted with buffered saline borate (BSB, 0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, 0.075 M NaCl, 1% w/v PVP, pH 8.45) (2 mg/ml) was carried out by incubation with 1 mg/ml PBS pH 7.4 of each enzyme at 55 °C over a period of 19 h while taking samples at various time points (0, 1, 2, 3 and 19 h). After SDS-PAGE analysis, E3 and E5 or E1, E3, E5 and E7 enzymes were selected for further experiments in pistachio and in

cashew, respectively. Such assays included the enzymatic digestion of whole nut paste (as opposed to soluble extract) under sonication (ultrasound disruption). The whole nut paste was prepared by mixing pistachio and cashew defatted flour with distilled water (0.5 g/ml) and kept at −20 °C. Cashew and pistachio paste was subjected to ultrasound equipment consisting of a seven frequency multiSONIK-2 Ultrasonic Generator (Blackstone-Ney Ultrasonics, Jamestown, NY, USA) and controlled according the method described by Easson et al. (2015). A thermocouple system was installed in conjunction with the ultrasound instrument to maintain a steady temperature of 55 °C. Fifteen ml plastic tubes, containing 0.5 g of untreated pistachio or cashew paste in 10 ml of distilled water followed by 110 μL of protease enzymes (1 mg/ml, Amano Enzyme, Swizerland), were sonicated for 1 h for pistachio or 2 h for cashew at 55 °C and 40 kHz ultrasound frequency at 980 W of power. A 100 μL aliquot was removed at the beginning of the experiment prior to adding the protease enzyme (untreated, no E control), and at various time points, after sonication with enzymes, and boiled at 65 °C for 15 min in sample buffer and analyzed by SDS-PAGE and western blot as control sample. All the experiments were performed at least in duplicate and the figures are representative of these replicate experiments.

2.3. Protein electrophoresis and IgE immunoblot experiments

Protein electrophoresis of the defatted flours was carried out as previously described (Cabanillas, Maleki et al., 2012). Defatted flours from untreated and treated cashew and pistachio were dissolved in standard electrophoresis sample buffer. The same amount of protein (20 μg) calculated from LECO analysis from each sample was electrophoresed in 4–20% and 16% Tris-glycine gels. Proteins were visualized with GelCode Blue Stain Reagent (Thermo Scientific, Waltham, MA, USA). For western blot, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA, USA). After blocking with 2% non-fat milk in PBS (1 h at 37 °C), membranes were incubated overnight at 4 °C with individual sera from 7 patients with pistachio and cashew allergy (*P*1-P7) (1:10, 1:5 or 1:3 dilution) washed and then treated with Horseradish peroxidase (HRP) conjugated mouse anti-human IgE (1:10,000 dilution for 30 min at RT) (Sigma, Saint Louis, MO, USA). Detection of IgE-binding proteins was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). The signal was measured using CCD camera system (Fuji Photo Film Co., Ltd., Duluth, GA, USA).

2.4. ELISA inhibition assays

Polystyrene microtiter plates (Immulon 4 HBX, Thermo Scientific, Waltham, MA, USA) were coated with 50 μl/well of raw cashew or pistachio protein extracts (10 μg/ml or 0.5 μg/well in 0.1 M $NaHCO_3$) and incubated for 1 h at 37 °C. In parallel, a serum from a pistachio and cashew allergic patient (#5, 1:2 dilution) was preincubated with untreated raw (control) or enzymatically treated raw, boiled and autoclaved cashew or pistachio samples as inhibitors (final concentrations: 10, 5 and 2.5 μg/ml) 1 h at 4 °C with rocking. Serum preincubated with PBS was also included (un-inhibited serum). Wells were washed with PBS containing 0.5% Tween-20 and blocked with 250 μl/well of PBS containing 3% non-fat milk (1 h at 37 °C). The serum, preincubated with the different inhibitors, or un-inhibited serum was then added to the wells (50 μl/well) of the pre-coated ELISA plates for 1 h. After washing the wells, 50 μl/well of a Horseradish peroxidase (HRP) conjugated mouse anti-human IgE (1:1000 dilution) (Sigma, Saint Louis, MO, USA) were added and incubated for 1 h. After washing, the peroxidase reaction was developed with 100 μL of peroxidase substrate (SureBlue TM, KPL, Gaithersburg, MD, USA). After 30 min, the reaction was stopped with 100 μL of 4 N HCl, and the optical density (O.D.) was measured at 450 nm. The percentage of the decrease in IgE binding (or inhibition) was calculated with the formula: $[(1 - (AI/AN)] \times 100$ where AI is the absorbance value obtained from raw cashew or pistachio samples preincubated with inhibited sera (raw or enzymatically hydrolyzed samples) and AN is the absorbance value of raw samples (cashew or pistachio) incubated with sera in the absence of inhibitor. All tests were performed in triplicate and the graphs are representative of these experiments. To test any significant differences between untreated (control) and treated samples for each inhibitor concentration, the inhibition data were subjected to one-way analysis of variance (ANOVA) and the means were compared by Duncan's multiple range test using the Statgraphic Centurion XVI.I software (Statpoint Tech. Inc., Warrenton, VA, USA). The table below each graph (Fig. 4) shows the comparison by Duncan's multiple range test between means of untreated and treated samples for each inhibitor concentration. Means in the same column followed with the same superscript are not significantly different ($P > 0.05$).

2.5. Liquid chromatography, mass-spectroscopy (LC/MS/MS)

Cashew and pistachio proteins from untreated, boiled for 60 min and autoclaved samples (120 kPa, 30 min and 256 kPa, 30 min) were digested by enzymatic treatment with enzyme 7 (cashew) and enzyme 5 (pistachio). The digestion resistant protein bands were excised from the gel following electrophoretic separation and identified by LC/MS/MS. Proteins were in-gel digested with trypsin (Sequencing Grade Modified, Promega, Madrid, Spain) according to Shevchenko, Tomas, Havlis, Olsen, and Mann (2006). Briefly, excised gel bands were washed with 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v) and incubated then placed in 10 mM dithiothreitol in 100 mM NH_4HCO_3 at 56 °C for 45 min followed by alkylation of the reduced sulfhydryl groups with 55 mM iodoacetamide in 100 mM NH_4HCO_3 at 25 °C for 30 min in the dark. Proteins were digested by adding 50 μL of trypsin (130 μg/ml) and incubating at 37 °C overnight. Peptides were eluted in extraction buffer (1:2 (v/v) 5% formic acid/acetonitrile) incubating at 37 °C for 15 min and 0.1% trifluoroacetic acid was added for further analysis. The tryptic digests were analyzed via LC/MS/MS, using an Agilent 1260 LC system, an Agilent Chip-cube interface and an Agilent 6520 Q-TOF tandem mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Data files were transferred to an Agilent workstation equipped with Spectrum Mill software (Agilent Technologies, Santa Clara, CA, USA) which extracted, sequenced, and searched databases such as TrEMBLE and NCBI.

3. Results

3.1. Assessing the efficiency of enzymes to digest cashew and pistachio protein

Seven enzymes from Amano Enzymes Ltd. were used for digestion of pistachio and cashew proteins in order to reduce the IgE binding properties. These enzymes were chosen for their higher temperature stability and differences in enzymatic properties. These 7 enzymes (E1-E7 at 1 mg/ml each) were each incubated with raw pistachio and cashew extracts for various amounts of time in order to identify the enzymes with the highest digestion ability. Digestion of pistachio proteins is shown as an example of this selection in Fig. 1A. The left panel shows the SDS-PAGE analysis of pistachio BSB extracts subjected to the longest digestion time (19 h) for each enzyme. Enzyme 3 and 5 (E3 and E5) were determined to be the most efficient in digesting pistachio proteins and used in further experiments. In the middle panel, autoclaved pistachio BSB extract (120 kPa, 15 min) incubated with E3 or E5 (1 mg/ml each) for 19 h is shown. In order to enhance digestion and reduce the processing time, the digestion experiments with whole pistachio paste were carried out in the presence of ultrasound treatment for 1 h at a frequency of 40 kHz, temperature of 55 °C and power of 980 watts at 100%. Due to the differences in digestion products for E3 and

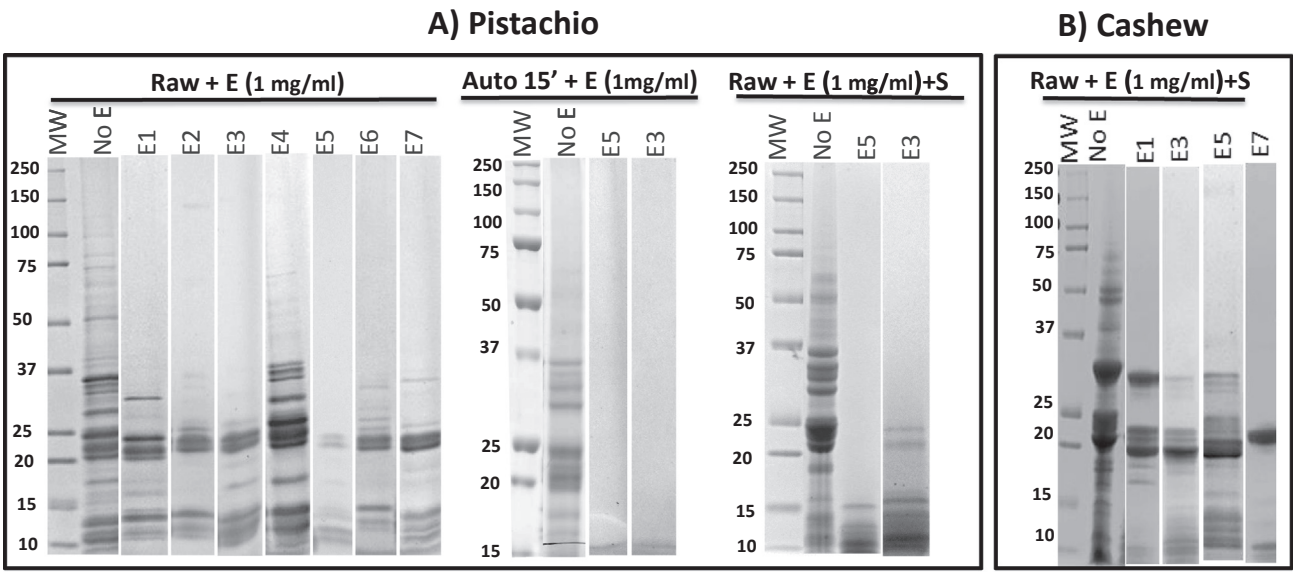


Fig. 1. A) SDS-PAGE analysis of the ability of various enzymes to digest pistachio proteins. Seven enzymes (E1-E7) were used at 1 mg/ml concentration to digest pistachio BSB extracts untreated (Raw, left panel) during 19 h without ultrasound treatment. Middle panel: Autoclaved pistachio BSB extract (120 kPa, 15 min) digested with enzymes E3 and E5 during 19 h without ultrasound treatment. Right panel: Digestion experiments of whole pistachio paste with enzymes E3 and E5 subjected to 1 h of ultrasound treatment (S). B) SDS-PAGE of digestion experiments of whole cashew paste with enzymes E1, E3, E5 and E7 subjected to 2 h of ultrasound treatment (S) Molecular weight marker (MW) and undigested pistachio or cashew (No E) are also shown. Each lane was loaded with 20 μg of protein. 4–20% Tris-glycine gels were used.

E5, E5 was selected for use in further experiments (Fig. 1A, right panel). Similar experiments were carried out to identify the most effective enzymes for the digestion of whole cashew paste with E1, E3, E5 and E7 (2 h, 40 kHz) and enzyme 7 (E7) was selected for further experiments (Fig. 1B). Multiple two-enzyme combinations of the seven enzymes were also used in digestion experiments for both cashew and pistachio extracts (data not shown). It was determined that the digestions were no more efficient with any two-enzyme combinations than the single enzymes selected for further experiments.

3.2. Electrophoretic characterization of processed cashew samples

The SDS-PAGE patterns of the proteins in paste made from untreated (control), boiled (boil), and autoclaved (AU) cashew before and after 2 h of simultaneous ultrasound treatment and digestion with the Amano Protease P 3DS (E7), are presented in Fig. 2A (lanes 1 to 7). Similar complex protein banding patterns were observed in raw, boiled and autoclaved at 120 kPa samples with bands ranging from 7 to 100 kDa. Cashew autoclaved at 256 kPa for 15 min and 30 min showed less defined stained bands and more smearing, most likely due to a combination of heat and pressure-induced aggregation and degradation of proteins. The protein migration patterns of untreated cashew (control) and thermally treated (boiling and autoclaving) after enzymatic digestion with E7 are also shown in Fig. 2A (lanes 8–14). The cashew protein patterns before and after enzymatic hydrolysis were very different with an important decrease in proteins higher than 25 kDa. Bands around 20 kDa and lower molecular weights were still present after enzymatic treatment of raw and boiled samples. However, many of the bands appeared to be digested with E7 in most of the autoclaved samples, as seen in lanes 12–14.

The samples were also analyzed for differences in IgE binding by immunoblot using individual sera from 6 patients (P1-P6) with clinical allergy to cashew (Fig. 2B). Previous studies have shown that certain physical processing can alter protein solubility affecting the extraction of soluble proteins by traditional protein extraction methods (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010), therefore defatted flours were directly solubilized in sample buffer and used for the SDS-PAGE and immunoblot analysis carried out in this study. Since the samples that had been autoclaved for 15 min (lanes 4 and 6) seemed to alter the protein profile less than the samples autoclaved for 30 min, they were not assessed for IgE binding by all patient sera. However, all blots contain raw, boiled 60 min and autoclaved (at 120 kPa, 30 min and 256 kPa, 30 min) samples before and after enzymatic treatment (lanes 1, 3, 5, 7, 8, 10, 12 and 14) for comparison purposes. Similar allergenic proteins were recognized by IgE in raw, boiled and autoclaved at 120 kPa cashew before enzyme treatment when sera from patients P1 to P5 were used in immunoblots (Fig. 2B). However, serum IgE from patient P6 recognized a different pattern of bands from 12 to 75 kDa in untreated cashew (lane 1) than in thermally processed cashew. Autoclaved cashew at 256 kPa (lanes 6 and 7) showed a similar IgE blot pattern to SDS-PAGE (Fig. 2A) with an overall reduction in the distinct IgE-reactive bands with increased time of treatment.

The results showed that numerous proteins from untreated and heat treated cashew samples were digested after 2 h of incubation with Amano Protease P 3DS (E7) under sonication; however, a few digestion resistant, IgE binding proteins could be visualized with sera from patients P1 (at and below 20 kDa, lanes 8, 10 and 12), P4 (10 kDa) and P5 (14 and 50 kDa). Autoclaved cashew proteins at 256 kPa showed higher susceptibility to digestion since no IgE-binding bands could be detected in 5 out of 6 sera used (lane 14).

3.3. Electrophoretic characterization of processed pistachio samples

The SDS-PAGE patterns of the proteins in paste made from untreated (control), boiled (boil), and autoclaved (AU) pistachio, before and after 1 h of simultaneous ultrasound treatment and digestion with the Amano Protease Protin (E5), are presented in Fig. 3A. Multiple bands can be seen in control sample with molecular weights between 6 and 100 kDa. Less defined bands were observed after autoclaving with an increase in protein aggregation and therefore higher molecular weight smears, particularly visible in samples autoclaved at 256 kPa (lanes 6 and 7). Digestion of treated samples with E5 enzyme (lanes 8–14) resulted in hydrolysates with an important decrease of higher MW proteins, above 15 kDa, and an increase of low molecular weight bands in the treated samples (lanes 9–14).

All samples were analyzed with IgE-immunoblot using a serum from 6 patients with clinical allergy to pistachio (P1, P2, P3, P4, P5 and P7). Fig. 3B shows the IgE-binding patterns to raw and processed pistachio

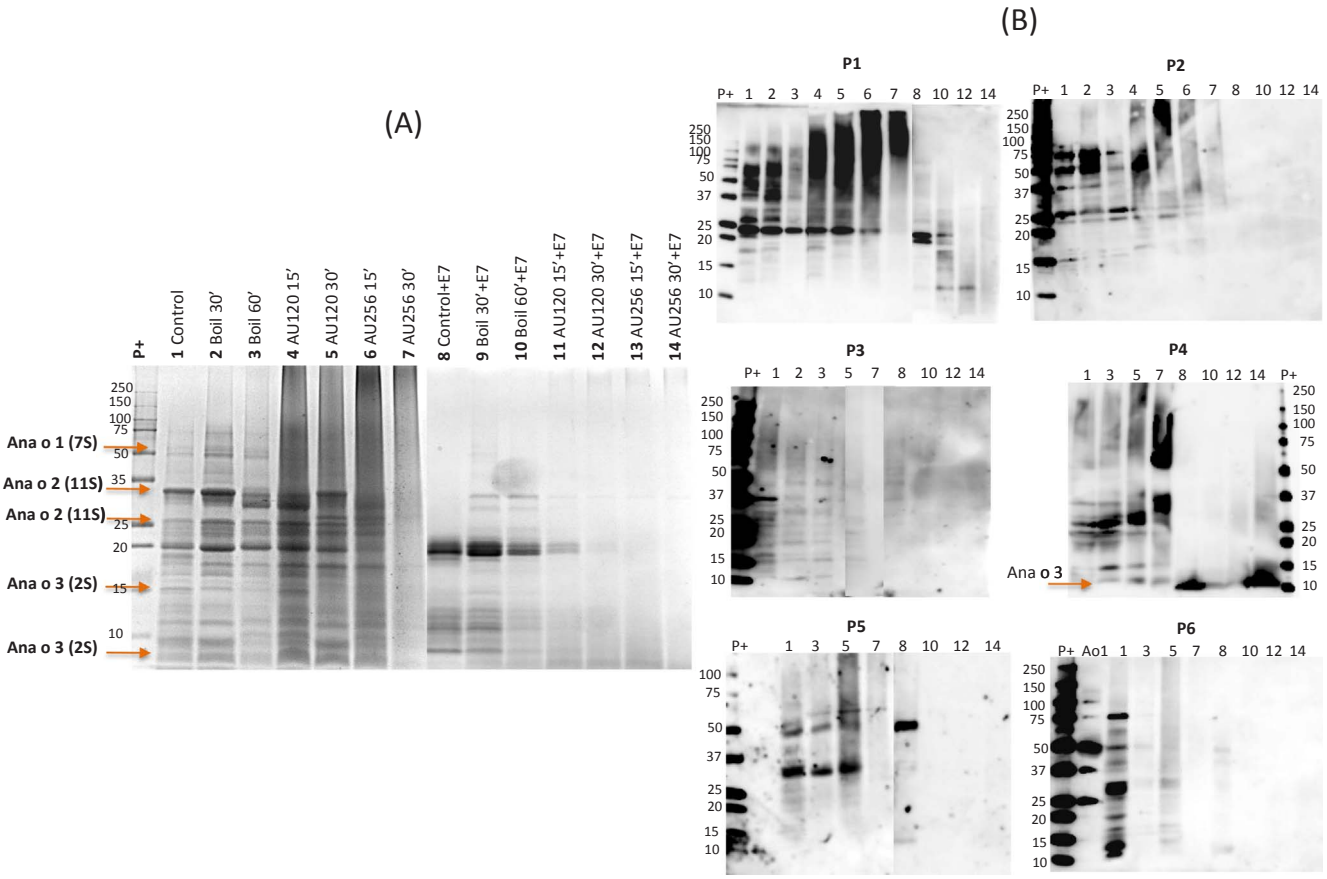


Fig. 2. SDS-PAGE (A) and IgE immunoblots (B) of untreated cashew paste (control; lanes 1 and 8) and processed cashew paste: boiled for 30 min (lanes 2 and 9) or 60 min (lanes 3 and 10); autoclaved at 120 kPa for 15 min (lanes 4 and 11) or 30 min (lanes 5 and 12); autoclaved at 256 kPa for 15 min (lanes 6 and 13) or 30 min (lanes 7 and 14) before and after incubation with enzyme 7 (Protease P, Amano 3DS), during sonication (2 h, 40 kHz). IgE immunoblots were carried out using individual sera from 6 patients allergic to cashew (P1-P6). Each lane was loaded with 20 μg of protein. 16% Tris-glycine gels were used.

samples before and after enzymatic treatment with E5. As mentioned above for cashew, only representative samples were subjected to immunoblots. Raw pistachio showed a pattern of IgE-binding to proteins in the range of 12–100 kDa. Pistachio IgE immunoreactivity appeared reduced with autoclaving at 256 kPa for several patient sera, but not after boiling or autoclaving at 120 kPa. Most proteins from untreated and processed pistachio samples were digested after 1 h of sonication in the presence of E5. Serum IgE from 2 out of 6 sera (P1 and P4) were still able to detect and bind to several proteins of varying molecular weights and smears as high as 75 kDa in the case of P4. No IgE reactive protein bands were detected after digestion of autoclaved pistachio at 256 kPa for all patient sera, except P4.

3.4. ELISA

In order to assess the IgE reactivity of cashew and pistachio hydrolysates, a competitive inhibition ELISA assay was carried out using serum from patient P5 with clinical allergy to pistachio and cashew. In this ELISA experiment, raw cashew or pistachio was used as a solid phase and raw and enzymatically treated raw, boiled and autoclaved cashew or pistachio samples were used as inhibitors. The percentage of the decrease in IgE binding was calculated as described in methods section and shown in Fig. 4A and B. The table below each graph shows the comparison by Duncan's test between means of untreated and treated samples for each inhibitor concentration. Raw cashew competed for IgE before and after hydrolysis with enzyme E7 (92.35% and 73.41%, respectively). However, the processed and digested cashew samples bound significantly lower IgE levels than the raw sample. Boiled (60 min) and autoclaved (256 kPa, 30 min) cashew hydrolysates samples inhibited only 24.16% of the IgE binding to raw cashew (Fig. 4A). Meanwhile, the effect was more pronounced with pistachio samples and all of the enzymatically treated samples. Raw as well as boiled and autoclaved pistachio were significantly weaker competitors for IgE binding compared to untreated raw pistachio (Fig. 4B). These findings are consistent with the reduction in IgE binding seen in the western blot analysis (Figs. 2B and 3B).

3.5. Proteins identified after enzymatic treatment of processed pistachio and cashew samples

Cashew and pistachio proteins from untreated, boiled for 60 min and autoclaved samples (120 kPa, 30 min and 256 kPa, 30 min) after digestion by treatment with E7 enzyme (cashew) and E5 enzyme (pistachio) were loaded in a 16% Tris-glycine gel in order to determine the extent of enzyme. The major bands were tryptic digested in order to carry out the analysis by mass spectroscopy (LC/MS/MS). Finally, twelve digestion resistant bands of pistachio and twelve digestion resistant bands in cashew were manually excised for LC-MS/MS identification (Fig. 5). Table 2 supp summarizes the polypeptide identification data of these pistachio and cashew samples. A peptide mass fingerprint search allowed us to identify all the resistant bands of pistachio (1–12) as Pis v 1, Pis v 2.01 or Pis v 5. The reason why the Pis v 1 peptides are so few is most likely due to the fact that the digested fragments, smaller than 8–10 kDa, ran off of the bottom of the gel. The bands # 4 and 7 had very few peptides identified and there were no peptides for Pis v 2 or Pis v 5 identified in band #10. Similar pattern is found with bands # 2, 5, 8, and 11 as well as for bands # 3, 6, 9 and 12. The bands # 13, 16, 19 and 22 (20 kDa) were identified as Ana o 2. and

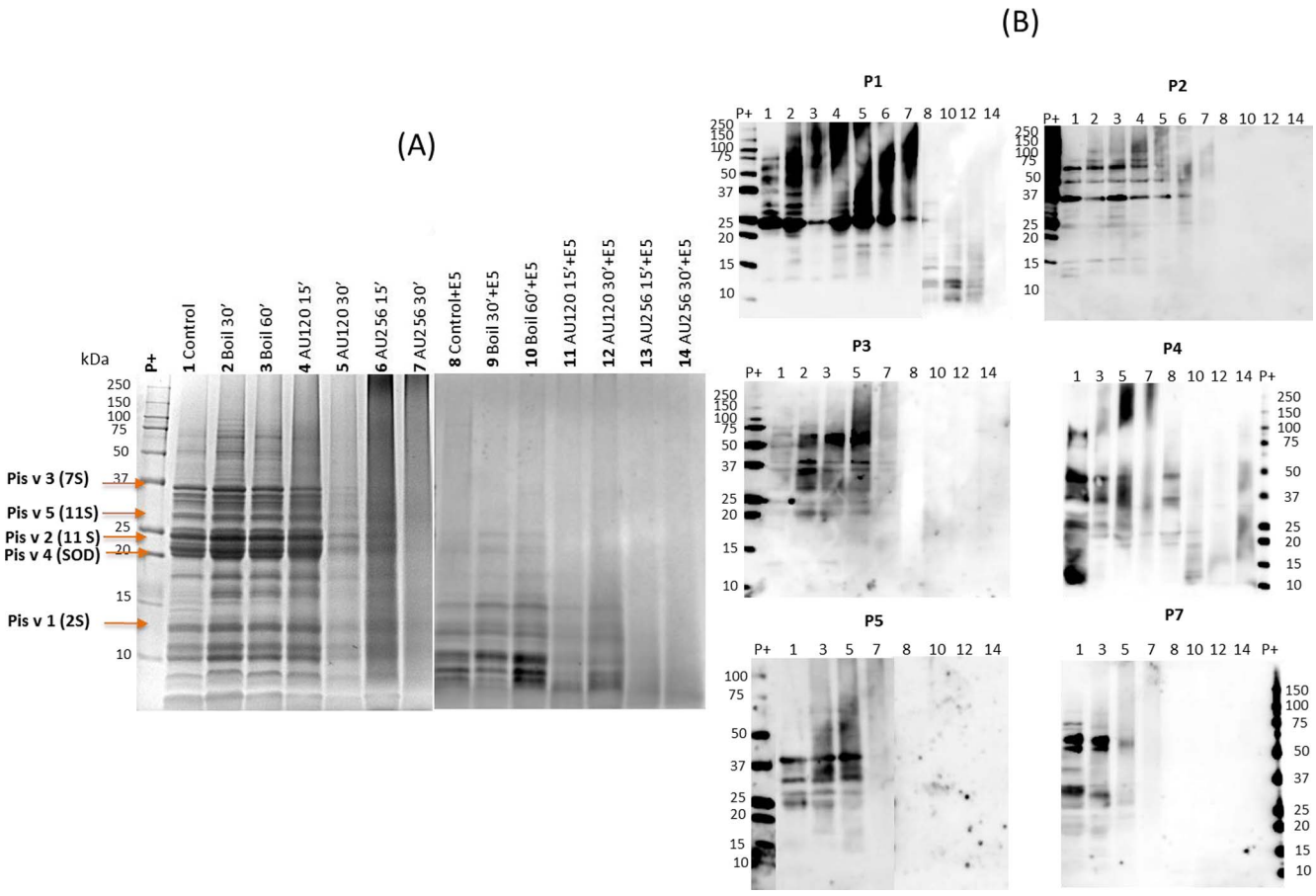


Fig. 3. SDS-PAGE (A) and IgE immunoblots (B) of untreated pistachio paste (control; lanes 1 and 8) and 6) and processed pistachio paste: boiled for 30 min (lanes 2 and 9) or 60 min (lanes 3 and 10); autoclaved at 120 kPa for 15 min (lanes 4 and 11) or 30 min (lanes 5 and 12); autoclaved at 256 kPa for 15 min (lanes 6 and 13) or 30 min (lanes 7 and 14) before and after sonication (1 h, 40 kHz) with enzyme 5 (Protin, Amano). IgE immunoblots were carried out using individual sera from 6 patients allergic to pistachio (P1-P7). Each lane was loaded with 20 μg of protein. 16% Tris-glycine gels were used.

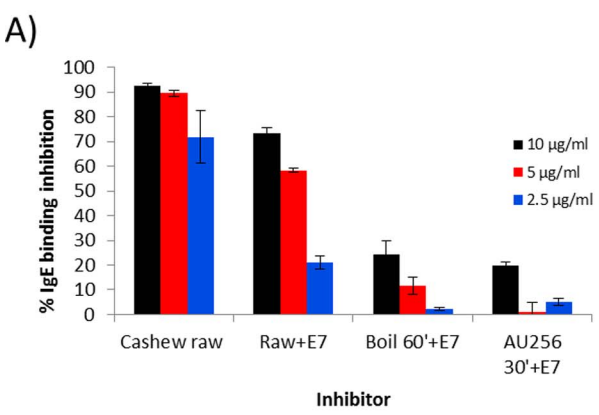
the bands # 17, 20 and 23 were also identified as Ana o 2, except one peptide which was identified as Ana o 1 (this could be an artifact, but the scores are around 83–84). Ana o 3 is detected in bands # 14 and 17, but not in bands # 20 and 23, suggesting that is degraded into smaller fragments. This is confirmed because Ana o 3 fragments are found in bands # 15, 18, 21 and 24. These findings show that the proteins are getting degraded over thermal treatment and enzymatic digestion and when autoclaved samples at 256 kPa are digested (bands # 10, 11, 12, 22, 23 and 24) only very few resistant peptides could be identified indicating that some fragments of pistachio and cashew allergens survive even thermal and enzymatic treatment.

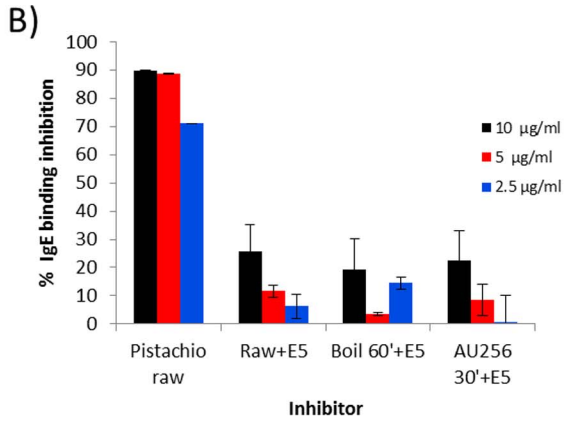
4. Discussion

In the present study, we have shown for the first time that IgE binding capacity of cashew proteins substantially decreased after pressure treatment at 256 kPa, 138 °C. We also found that some IgE-binding proteins from cashew were stable following boiling and autoclaving at 120 kPa. In accordance with these results, Venkatachalam et al. (2008) found that simple food processing treatments (autoclaving at 121 °C, blanching, microwave heating, roasting, gamma irradiation and pH) may not be sufficient to eliminate or substantially reduce cashew nut allergenicity as Ana o 1, Ana o 2 and Ana o 3 survive the processing conditions tested. Our findings indicate that enzymatic digestion with Amano Protease P 3DS (E7) during 2 h of sonication reduced the number of bands recognized by IgE of cashew allergic patients sera. Ana o 2 (36 kDa and 20 kDa bands) recognition was almost abolished but still some digestion resistant proteins were recognized by 50% of sera tested (Fig. 2B) and Ana o 3 (~8 kDa band) is still detected by IgE in one of the immunoblots (Fig. 2B). However, a combination of both, enzymatic hydrolysis under sonication and thermal treatment, was a more effective method to reduce the allergenic reactivity of cashew. The results of competitive inhibition ELISA (Fig. 4A) confirmed the greater reduction of IgE binding capacity of boiled and autoclaved cashew allergenic proteins compared to raw cashew.

Similarly to cashew, IgE immunoreactivity of pistachio was strongly reduced with thermal treatment with high pressure (autoclaving at 256 kPa), but not after boiling or autoclaving at 120 kPa. These findings are also in agreement with our previous results with other tree nuts and legumes (Cabanillas, Maleki et al., 2012; Cabanillas et al., 2014; Cuadrado et al., 2009; Álvarez-Álvarez et al., 2005). In contrast, the influence of enzymatic digestion on pistachio allergens was more pronounced. Most of immunoreactive proteins from untreated and processed pistachio samples were digested with Protin (E5) in conjunction with 1 h of sonication (Fig.3B). In this case, the antigenicity of pistachio was almost abolished after digestion of raw and thermally treated samples (Fig.4B). After the enzymatic treatment, the results of protein identification by MS analysis (Fig. 5 and Table 2 supp) indicate that the allergens are being degraded over thermal treatment and enzymatic digestion. Enzymatic digestion of processed samples produced few resistant peptides indicating that some fragments of pistachio and cashew allergens survive even following thermal and enzymatic treatments.

Cabanillas et al. (2010, 2012) concluded that sequential hydrolysis of lentil and peanut by endo- and exoproteases results in an important proteolytic destruction of IgE-binding proteins. Cabanillas et al. (2014) demonstrated higher susceptibility to digestion of pressure treated





% IgE binding inhibition

Inhibitor	2.5 µg/ml	5 µg/ml	10 µg/ml
Cashew raw	71.9^{a}	89.5^{a}	92.3^{a}
Raw+E7	20.9^{b}	58.3^{b}	73.4^{b}
Boil 60'+E7	2.2^{c}	11.7^{c}	24.2^{c}
AU256 30'+E7	5.2^{c}	1.2^{d}	19.8^{c}

% IgE binding inhibition

Inhibitor	2.5 µg/ml	5 µg/ml	10 µg/ml
Pistachio raw	71.0^{a}	88.7^{a}	89.9^{a}
Raw+E7	6.2^{bc}	11.7^{b}	25.7^{b}
Boil 60'+E7	14.5^{b}	3.6^{c}	19.2^{b}
AU256 30'+E7	0.5^{c}	8.6^{bc}	22.4^{b}

Fig. 4. Competitive inhibition ELISA of IgE binding to immobilized raw cashew (A) and raw pistachio (B) by three different concentrations (10 µg/ml, 5 µg/ml; 2.5 µg/ml) of untreated raw (control) or enzymatically treated raw, boiled and autoclaved cashew or pistachio samples as inhibitors. Panels A and B show the percentage of inhibition of IgE binding (mean ± SD) by the indicated concentrations of cashew and pistachio samples. The table below each graph shows the comparison by Duncan's multiple range test between means of untreated and treated samples for each inhibitor concentration. Means in the same column followed with the same superscript are not significantly different. ($P < 0.05$).

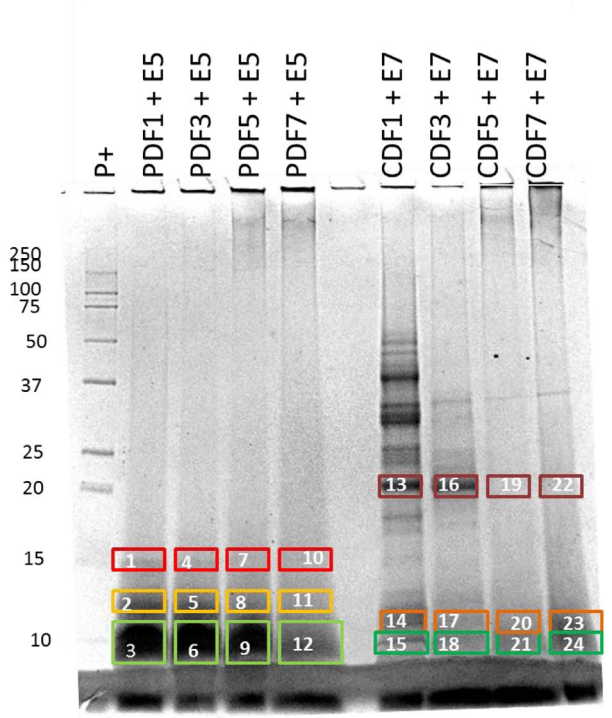


Fig. 5. SDS-PAGE (16% Tris-glycine) of untreated, boiled (60 min) and autoclaved (120 kPa, 30 min and 256 kPa, 30 min) pistachio paste (PDF1, PDF3, PDF5, PDF7) and cashew paste (CDF1, CDF3, CDF5, CDF7) after incubation with enzyme 5 (1 h, 40 kHz) and enzyme 7 (2 h, 40 kHz), respectively. Twelve digestion resistant bands of pistachio and twelve digestion resistant bands in cashew (squared) were manually excised for LC-MS/MS identification. Each lane was loaded with 20 µg of protein.

walnut proteins. That is in agreement with the present results with cashew and pistachio showing that enzymatic hydrolysis is more efficient on autoclaved cashew and pistachio samples.

In conclusion, the results indicate that autoclave treatment and enzymatic hydrolysis under sonication separately induced a measurable reduction of the IgE binding properties IgE binding of pastes (as opposed to soluble extracts) made from treated cashew and pistachio nuts, being more effective with pistachio allergens. However, thermal treatment (boiling and autoclaving) combined with enzymatic digestion is necessary to substantially reduce IgE binding to cashew allergens. Such studies are important as a preliminary step prior to clinical oral challenge trials to confirm a possible decrease in allergenicity of these processed foods. The findings of this study identify treatment conditions to reduce or even abolish the allergenic reactivity of cashew and pistachio in order to produce hypoallergenic foods, which may be relevant for consumers, clinicians, regulatory agencies and food industry.

The use of processed cashew and pistachio with low IgE binding capacity might be a convenient strategy for tolerance induction. Evaluation of sensorial properties and clinical oral challenge trials are necessary to analyze organoleptic properties and to confirm the decrease of allergenicity of these processed nut pastes.

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Note

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.08.120.

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Appendix 2. Supplementary Material

Table 1 supplementary material. Immunological and clinical information of the 7 patients allergic included in this study.

Patient no.	Age/Sex	IgE total (kU/L)	IgE cashew (kU/L)	IgE pistachio (kU/L)	IgE peanut (kU/L)	Symptoms
# 1	58/F	13665	4.5	ND	26.1	C, EB, eye ulcers, itchy nose, CT, HF.
# 2	65/M	71000	ND	ND	>100	Severe eczema, hearing loss, pruritus, EB.
# 3	22/M	1200	17	8	>100	EB, TS, FS, LS, G, IM, H.
# 4	36/M	27.5	5.97	ND	8.45	Severe eczema.
# 5	26/M	362.9	10.1	5	>100	Anaphylaxis.
# 6	30/F	191	1.46	2.09	52.7	SOB, wheeze, severe asthma.
# 7	41/F	120	8.5	5.9	1.6	TS, CT, IT, IM, N, GI, V.

C, conjunctivitis; CT, chest tightness; EB, eye burn/itchy; FS, face swell; G, general itching; GI, gastrointestinal pain; H, hives; HF, hay fever; IM, itchy mouth; IT, itchy throat; LS, lip swell; N, nausea; SOB, shortness of breath; TS, throat swell; V, vomiting.
ND, not determined.

Table 2 supplementary. Cashew and pistachio proteins from untreated, boiled for 60 min and autoclaved samples (120 kPa, 30 min and 256 kPa, 30 min) treated by enzymatic digestion with enzyme 7 (cashew) and enzyme 5 (pistachio) and identified by LC/MS/MS.

Band No.	Protein identification	Mass (Da)	Matched peptides
1	Pis v 2.01/Pis v 5 (11S *P.vera*)	54,465	10
1	Pis v 1 (2S *P.vera)*	17,290	1
2	Pis v 2.01/Pis v 5 (11S *P.vera*)	54,465	22
2	Pis v 1 (2S *P.vera)*	17,290	1
3	Pis v 2.01/Pis v 5 (11S *P.vera*)	54,465	19
3	Pis v 1 (2S *P.vera)*	17,290	1
4	Pis v 2.01/Pis v 5 (11S *P.vera*)	54,465	3
5	Pis v 2.01/Pis v 5 (11S *P.vera*)	54,465	28
5	Pis v 1 (2S *P.vera)*	17,290	1
6	Pis v 2.01/Pis v 5 (11S *P.vera*)	54,465	21
6	Pis v 1 (2S *P.vera)*	17,290	1
7	Pis v 2.01/Pis v 5 (11S *P.vera*)	54,465	1
8	Pis v 2.01/Pis v 5 (11S *P.vera*)	54,465	6
9	Pis v 2.01/Pis v 5 (11S *P.vera*)	54,465	11
9	Pis v 1 (2S *P.vera*)	17,290	1
11	Pis v 2.01/Pis v 5 (11S *P.vera*)	54,465	2
12	Pis v 2.01/Pis v 5 (11S *P.vera*)	54,465	8
12	Pis v 1 (2S *P.vera*)	17,290	1
13	Ana o 2 (11S *A. occidentale*)	51,996	13
14	Ana o 2 (11S *A. occidentale*)	51,996	7
14	Ana o 3 (2S *A. occidentale*)	16,335	2
15	Ana o 2 (11S *A. occidentale*)	51,996	6
15	Ana o 3 (2S *A. occidentale*)	16,335	3
16	Ana o 2 (11S *A. occidentale*)	51,996	15
17	Ana o 1 (7S *A. occidentale*)	61,841	1
17	Ana o 2 (11S *A. occidentale*)	51,996	6
17	Ana o 3 (2S *A. occidentale*)	16,335	2
18	Ana o 2 (11S *A. occidentale*)	51,996	9
18	Ana o 3 (2S *A. occidentale*)	16,335	2
19	Ana o 2 (11S *A. occidentale*)	51,996	8
20	Ana o 1 (7S *A. occidentale*)	61,841	1
20	Ana o 2 (11S *A. occidentale*)	51,996	6
21	Ana o 1 (7S *A. occidentale*)	61,841	2
21	Ana o 2 (11S *A. occidentale*)	51,996	5
21	Ana o 3 (2S *A. occidentale*)	16,335	1
22	Ana o 2 (11S *A. occidentale*)	51,996	5
23	Ana o 1 (7S *A. occidentale*)	61,841	1
23	Ana o 2 (11S *A. occidentale*)	51,996	6
24	Ana o 1 (7S *A. occidentale*)	61,841	2
24	Ana o 2 (11S *A. occidentale*)	51,996	6
24	Ana o 3 (2S *A. occidentale*)	16,335	2

Anexos

LC-MS/MS

Se han identificado los péptidos resistentes al tratamiento de autoclave 138°C 30 minutos de anacardo y pistacho, mediante cromatografía líquida acoplada a espectrometría de masas (LC-MS/MS). El tampón de electroforesis, así como los reactivos necesarios para la realización de los geles de acrilamida, se filtraron con tamaño de poro de 0.22 μm o se autoclavaron para obtener condiciones adecuadas de higiene y esterilidad, evitando la contaminación del gel con pelo, piel o fibras de tejidos. Con un bisturí estéril, se recortó una banda de unos 2 mm, entre los pesos moleculares de 15 y 10 kDa (Figura A1) y se dividió en cubos de unos 10 mm^3. Los cubos de gel fueron digeridos con tripsina y analizados mediante LC-MS/MS por el servicio de proteómica de la Universidad de Bonn (Thermo Fisher Scientific, Bremen, Germany). En las tablas A1 y A2 se muestran los péptidos encontrados con mayor abundancia en anacardo y pistacho. En anacardo se añade el número del péptido inmunoreactivo descrito previamente en la bibliografía (como #núm.). En pistacho no se han descrito estos epítopos hasta la fecha. Por ello, y dada la homología existente entre los alérgenos de pistacho y anacardo, se ha realizado un alineamiento de las secuencias aminoacídicas de alérgenos de ambos frutos secos, disponibles en las bases de datos, para comparar la secuencia de aminoácidos de algunos de los péptidos encontrados con mayor abundancia en pistacho con la de epítopos descritos como inmunoreactivos en anacardo.

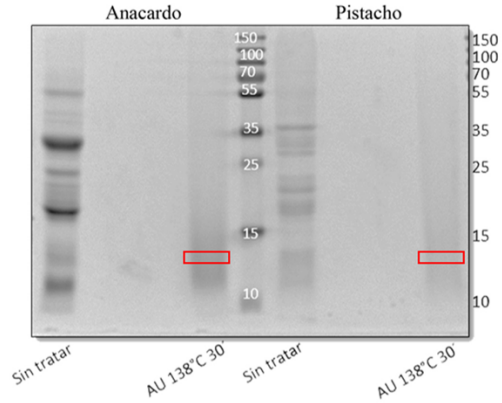

Figura A1. Electroforesis monodimensional SDS-PAGE con tinción Coomassie (condiciones reductoras, 12%, 20 μg) de extractos proteicos de anacardo y pistacho sin tratar y tratado por autoclave a 138°C/2.56 atm 30 min. Los cuadros rojos marcan las bandas recortadas del gel y analizadas por LC-MS/MS. El marcador de peso molecular es Page Ruler Prestained Ladder (Thermofisher).

Tabla A1. Identificación de péptidos resistentes al tratamiento de autoclave 138°C/2.56 atm 30 min en anacardo por LC-MS/MS. *Número de epítopo immunoreactivo de Ana o 2 y Ana o 3 (#) descritos previamente por Wang *et al,* 2003 y Robotham *et al,* 2005 respectivamente. aa: amino ácido

Accesion	Péptidos	Primer aa	IgE-epítopo lineal *
Ana o 2 AAN76862.1	[N]PKDVFQQQQQHQSR[G]	182	#11
	[D]TELLAEAFQVDER[L]	205	--
	[D]PARADIYTPEVGR[L]	289	--
	[R]LTTLNSLNLPILK[W]	302	--
	[R]EGQMLVVPQNFAVVK[R]	369	#19
	[R]FEWISFK[T]	390	#20
	[R]TSVLGGMPEEVLANAFQISR[E]	410	#21
Ana o 3 AAL91665.1	[R]QLQQQEQIKGEEVR[E]	98	#13/14
	[R]ICSISPSQGCQFQSSY[-]	123	#16

Tabla A2. Identificación de péptidos resistentes al tratamiento de autoclave 138°C/2.56 atm 30 min en pistacho por LC-MS/MS. Se destacan en negrita los péptidos identificados en pistacho con secuencia similar a la de epítopos de relevancia inmunoreactiva descritos previamente en proteínas homólogas de anacardo (representado en Figura A2).

Accesion	Péptidos	Primer aa
Pis v 1 ABG73108.1	[R].**MCNISPSQGCQFSSPY**.[W]	131
	[K]LQELYETASELPR[M]	118
	[K].**RQQQQGQFRGEK**.[L]	106
	[R].CQNLEQMVK.[R]	97
	[K].**HCQMYVQQEVQKS.**[Q]	52
Pis v 2.0101 ABG73109.1	[R].SETTIFAPGSSSQR.[K]	465
	[R].ALPLDVIK.[N]	440
	[R].**FLQLSAK**.[K]	340
	[R].VSSVNALNLPILR.[F]	327
	[K].**FVLGGSPQQEIQGSGQSR**.[S]	195
Pis v 2.0201 ABG73110.1	[R].SEMTIFAPGSR.[S]	456
	[R].**FLQLSVEK**.[G]	331
	[R].VTSINALNLPILR.[F]	318
	[R].**VKEDLQVLSPQR**.[Q]	268
	[Q].SFNIDTQLVK.[K]	244
	[K].**FVLGGSPQQEIQGGGQ**.[S]	200
Pis v 3 ABO36677.1	[K].STGTFNLFK.[K]	325
	[K].LFEKQDEGAIVK.[A]	289
	[R].VKTEQGKVVVLPK.[F]	140
	[K].**GREQEEEEEEEWGSGR**.[G]	40
	[R].**QYDEEQKEQCAK**.[G]	18
Pis v 5 ACB55490.1	[K].IKFNNEQPTLSSGQSSQ.[Q]	451
	[R].**AMPEEVLANAFQISR**.[E]	431
	[R].AMISPLAGSTSVLR.[A]	417
	[D].PSRSDIYTPEVGR.[I]	305
	[R].**GFESEEESEYER**.[G]	269
	[R].CAGVAVAR.[H]	66

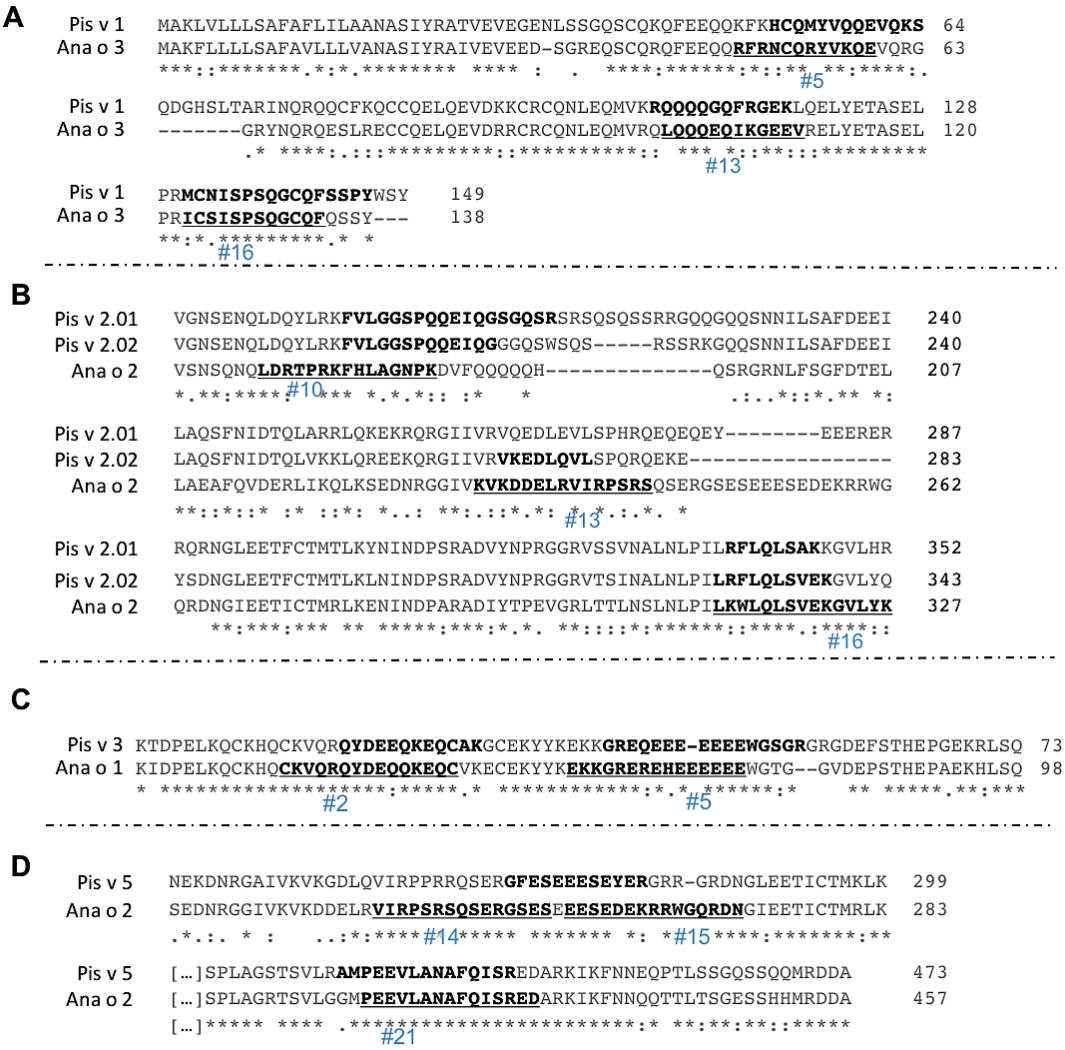

Figura A2. Alineamientos de las secuencias aminoacídicas parciales de alérgenos de pistacho y anacardo, (Clustal Ω, secuencias disponibles en la base de datos *NCBI*). Se marcan algunos de los péptidos identificados en pistacho con mayor abundancia (señalados en negrita, tabla A2) y que incluyen aminoácidos idénticos o similares a los presentes en epítopos de proteínas homólogas de anacardo (señalados en negrita y subrayado). A) alineamiento parcial de Pis v 1 y Ana o 3 (albúminas 2S); epítopos descritos en anacardo por Robotham *et al,* 2005. B) alineamiento parcial de Pis v 2 (ambas isoformas) y Ana o 2 (leguminas 11S); epítopos descritos en anacardo por Wang *et al,* 2003. C) alineamiento parcial de Pis v 3 y Ana o 1 (vicilinas 7S); epítopos descritos en anacardo por Wang *et al,* 2002. D) alineamiento parcial de Pis v 5 y Ana o 2 (leguminas11S); epítopos descritos en anacardo por Wang *et al,* 2003). * *amino ácidos idénticos; . y : amino ácidos similares; - gaps.*

#Numeración de los epítopos de acuerdo a los autores.

Digestión enzimática individual con pepsina y tripsina

Se ha determinado la influencia de los tratamientos térmicos y de presión (cocción 60 y autoclave 121°C 30 minutos) sobre la susceptibilidad a la digestión enzimática con peptidasas propias del aparato digestivo, la pepsina y la tripsina (P6887 y T1426 respectivamente, Sigma-Aldrich, St.Louis, EEUU). El análisis se ha realizado por electroforesis monodimensional SDS-PAGE al 12% de acrilamida, cargando 5 µg (Figura A3). Las digestiones enzimáticas se han realizado de manera individual sobre extractos proteicos de pistacho y anacardo control, cocción 60 y autoclave 121°C 30 minutos, recogiendo muestras antes de la digestión (t=0) y varios tiempos de digestión con pepsina (se muestra tras 15 y 90 minutos) o tripsina (30 minutos y 16 horas). Los extractos proteicos de anacardo y pistacho fueron incubados a una concentración final de 0.4 mg/ml, en presencia de pepsina a 0.01 µM en una solución de 84 mM HCl, 35 mM de NaCl pH 1.2, simulando el fluido gástrico (SGF). Se empleó 200 mM de $NaHCO_3$ para parar la reacción de digestión enzimática. En el caso de la tripsina, los extractos, a la misma concentración, se incubaron en una solución 50 mM Tris BASE, 1 mM EDTA pH 8.3 y tripsina al 0.042 µM (SIF) y la reacción se paró calentando las muestras a 80^{o}C 5 minutos. Todos los tubos y reactivos se mantuvieron atemperados a 37^{o}C.

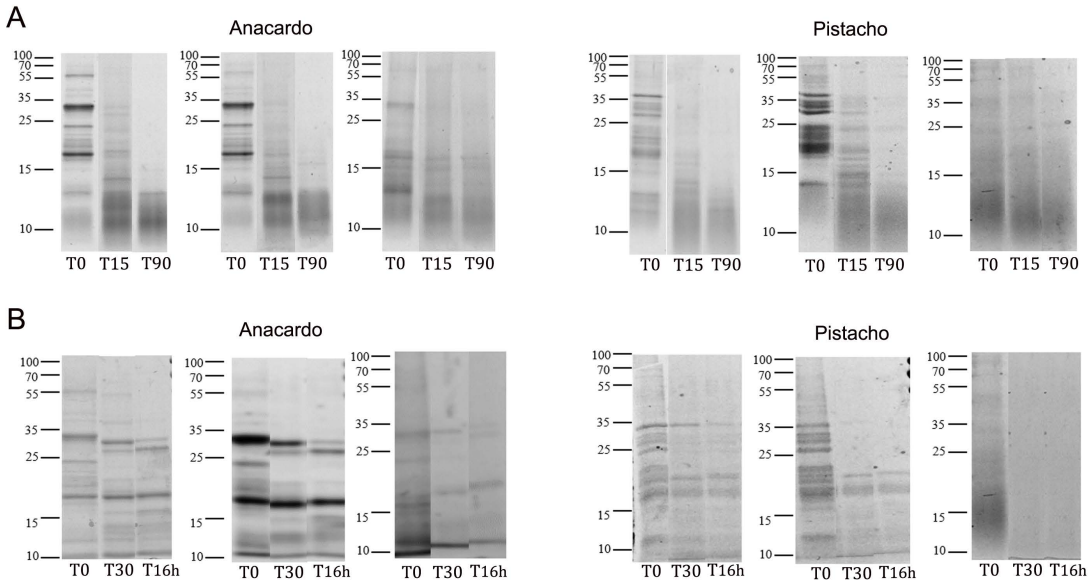

Figura A3. Digestibilidad *in vitro* de extractos proteicos de anacardo y pistacho mediante pepsina (A) y tripsina (B). Análisis por SDS-PAGE (condiciones reductoras, 12%, 5 µg) del transcurso de la digestión enzimática de muestras de anacardo y pistacho control, cocidas 60' y autoclavadas a 121^{o}C 30'.

ARTÍCULO 3

INFLUENCIA DEL PROCESADO TÉRMICO/DE PRESIÓN SOBRE EL CONTENIDO FENÓLICO, ACTIVIDAD ANTIOXIDANTE Y PROPIEDADES FUNCIONALES DE HARINAS PISTACHO, ANACARDO Y CASTAÑA

INFLUENCE OF THERMAL/PRESSURE PROCESSING ON THE PHENOLIC CONTENT, ANTIOXIDANT ACTIVITY AND FUNCTIONAL PROPERTIES OF PISTACHIO, CASHEW AND CHESTNUT FLOURS

Los frutos secos son una fuente rica en compuestos nutricionales con un efecto beneficioso para la salud. Sin embargo, en individuos sensibilizados, los frutos secos pueden causar graves reacciones alérgicas. El tratamiento térmico y de presión provoca un descenso en las propiedades de unión a IgE de pistacho, anacardo y castaña. El objetivo de este estudio ha sido evaluar el efecto del procesado basado en calor y/o presión en el contenido de proteína total, compuestos fenólicos (antocianinas, flavonoles, esteres tartáricos y fenoles totales), la actividad antioxidante y las propiedades funcionales de harinas de pistacho, anacardo y castaña. La cocción disminuyó el contenido de fenoles totales, flavonoles y esteres tartáricos en pistacho y anacardo, pero no afectó en castaña. El procesado térmico combinado con presión indujo un aumento del contenido en compuestos fenólicos. La actividad antioxidante no resultó apenas afectada por las condiciones más severas de autoclave (138°C, 2.56 atm, 30 min) e incluso sufrió un incremento en castaña. La actividad antioxidante, se determinó por los ensayos dimetil-p-fenilenediamina (DMPD) y por capacidad de absorbancia de radicales libres (ORAC), y está positivamente correlacionada con la cantidad de compuestos fenólicos. Los tratamientos de calor y presión (cocción y autoclave) producen un incremento de la capacidad de retención y absorción de agua en los tres frutos secos analizados, y la capacidad de absorción de aceite aumenta en harinas desengrasadas de pistacho y anacardo procesado. El procesado afectó negativamente a la capacidad emulsionante y espumante de pistacho, anacardo y castaña, así como la capacidad de gelificación en este último material. De esta forma, combinando calor y presión pueden obtenerse harinas de pistacho, anacardo y castaña, con propiedades alergénicas reducidas y que mantengan su contenido en fenoles y actividad antioxidante.

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INFLUENCE OF THERMAL/PRESSURE PROCESSING ON THE PHENOLIC CONTENT, ANTIOXIDANT ACTIVITY AND FUNCTIONAL PROPERTIES OF PISTACHIO, CASHEW AND CHESTNUT FLOURS

Abreviations (as foonotes):

DMPD, dimethyl-ρ-phenylenediamine; **DW,** dry weight; **EA,** emulsifying activity; **ES,** emulsifying stability**; FC,** foaming capacity; **FS,** foaming stability; **LGC,** least gelation concentration; **OHC,** oil holding capacity; **ORAC**, oxygen radical absorbance capacity, **RT**, room temperature; **WAC**, water absorption capacity; **WHC**, water holding capacity; .

Abstract

Tree nuts are a rich source of nutritional compounds with beneficial effect on health. However, in sensitized subjects, tree nuts can cause severe allergic reactions. Foods properties can be modified by processing and thermal/pressure treatment was able to decrease IgE-binding properties of pistachio, cashew and chestnut flours. The objective of this study was to evaluate the effect of processing based on heat and/or pressure on protein and phenolic compounds (anthocyanins, flavonols, tartaric esters and total phenols) content, antioxidant activity and functional properties of pistachio, cashew and chestnut flours. Boiling decreased total phenols, flavonols and tartaric esters content in pistachio and cashew but did not affect in chestnut. Phenolic compounds content was increased in pistachio, cashew and chestnut after heat combined with pressure processing. Antioxidant activity resulted almost not reduced by harshest autoclave condition (138°C, 2.56 atm, 30 min) and even, was increased in chestnut. The antioxidant activity, determined by dimethyl-ρ-phenylenediamine (DMPD) as well as oxygen radical absorbance capacity (ORAC) assays, was positively correlated with phenolic compounds content. Moist thermal and pressure treatments (boiling and autoclaving) increased

water holding capacity (WHC) and water absorption capacity (WAC) in the three analysed tree nuts, and oil holding capacity (OHC) raised in processed pistachio and cashew defatted flours. Emulsifying and foaming capacities of pistachio, cashew and chestnut were negatively affected by processing as well as chestnut gelation capacity. Therefore, pistachio, cashew and chestnut flours with reduced allergenic properties and maintaining their phenolic content and antioxidant activity can be obtained by combining heat and pressure processing.

Keywords: Tree nuts; Phenols content; Antioxidant activity; Functional properties; Thermal processing; Pressure processing.

1. Introduction

Tree nuts are a good source of macronutrients such as unsaturated fatty acids, proteins, vitamins, minerals, fibre and polyphenols, with very high antioxidant potential, among other properties (Kornsteiner, Wagner, & Elmadfa, 2006; Vinson & Cai, 2012). Nuts have been proposed as an essential ingredient for an optimal diet, especially for preventing heart diseases, diabetes type II and weight loss (Ribeiro, da Silva, de Almeida, Hermsdorff, & Alfenas, 2017; Ros, 2010). Consumption of Anacardiaceae nuts (pistachio and cashew) has experienced an important increase in the last years, due to their organoleptic quality and health benefits (Ros, 2010; Schulze-Kaysers, Feuereisen, & Schieber, 2015). They are a rich source of protein, carbohydrate and triglycerides, usually consumed roasted and salted, being worldwide appreciated as snacks. Chestnuts are one of the tree nuts with higher content of antioxidants, only after walnut and pecan nut, low fat content, high carbohydrates, fibre and vitamins (Blomhoff, Carlsen, Andersen, & Jacobs, 2006; Borges, Gonçalves, de Carvalho, Correia, & Silva, 2008; Braga, Rodrigues, & P.P. Oliveira, 2014). These nuts can be also included in a wide range of processed foods (cookies, ice cream, sauces, sweets, yogurt…). In recent years, flour from some seeds and nuts, as chestnut, rice or legumes, has aroused interest as substitutes of wheat flour for gluten-free formulations (Arribas et al., 2017; Demirkesen, Mert, Sumnu, & Sahin, 2010; Foschia, Horstmann, Arendt, & Zannini, 2017). Phenolic compounds are secondary metabolites present in all plants in different concentration, depending on the food source. Consumption of foods with high content of polyphenols is beneficial to health, regarding anti-cancer, anti-microbial and anti-inflammation properties (Cardador-Martínez et al., 2014; Yao et al., 2004). Free radicals are naturally released by cellular metabolism, and can attack many molecules as proteins, DNA or lipids. Free radical-based damage is connected to serious diseases, as Parkinson, heart attacks or Alzheimer (Sochor et al., 2010). Protective system of organisms is based on antioxidant activity of specific enzymatic and non-enzymatic

compounds. Antioxidant activity from phenolic compounds serves as free radical scavengers, reducing the risk to suffer cancer and other diseases (Cardador-Martínez et al., 2014; Sochor et al., 2010). The most important phenolic compounds include flavonoids, phenolic acids and less abundant stilbenes and lignans (Czubinski & Dwiecki, 2017; Ozdal, Capanoglu, & Altay, 2013).

Tree nuts are also associated to food allergy, being the most common offending foods in Spain after fruits (Fernández Rivas, 2009). Food allergies affect around 6–8% of children and approximately 2% of the general population, but specific prevalence is difficult to establish, being variable depending on the country, the individual and the food ingredient (Weinberger & Sicherer, 2018). Allergy to pistachio and cashew is responsible of severe symptoms and even anaphylactic reactions that can be lethal. Cashew, together to peanut, leads the cases of mortality for anaphylaxis in UK and USA (Costa, Silva, Vicente, & Oliveira, 2017; Ho, Wong, & Chang, 2014; Mendes, Costa, Vicente, Oliveira, & Mafra, 2016). Allergy to chestnut is mainly associated to latex-fruits syndrome, although chestnut allergic patients without associated latex hypersensitivity has been also described, showing different sensitization pattern (Sánchez-Monge et al., 2006). Since specific therapy for sensitized patients does not exist, complete avoiding of allergenic ingredients is the only advice to prevent severe anaphylactic reactions. Thermal and non-thermal treatments are commonly used in industry to improve food quality and, ensure food safety and to improve organoleptic properties for consumption. Many research studies have established that some technological treatments might affect structure and/or solubility of proteins and, consequently, the IgE binding capacities from allergens in foods (Álvarez-Álvarez et al., 2005; Cabanillas et al., 2012; Cabanillas et al., 2014; Cabanillas & Novak, 2017; Cuadrado et al., 2009). In pistachio, cashew and chestnut, heat combined with pressure processing (autoclave) has been successfully applied to decrease their IgE reactivity (Cuadrado et al., 2018; De las Cuevas, Garcia-Granero, Barranco, Crespo, &

Dieguez, 2018; Giménez, De La Cruz, Barranco, Crespo, & Dieguez, 2018; Sanchiz et al., 2018). Ingredients with probed reduced allergenicity can be used as additive in hypoallergenic food products and even for immunotherapy purposes in sensitized patients (Ogawa, Samoto, & Takahashi, 2000; Vickery et al., 2014).

Technological processing can influence not only the protein content but also on the protein functional properties, and the effect is variable depending on the protein denaturation as well as on other natural endogenous nutritional components in the foods (Aguilera, Esteban, Benítez, Mollá, & Martín-Cabrejas, 2009). Thus, analysis of nutritionally important components such as proteins and their functionality, phenolic compounds and their antioxidant activity, and the impact of processing on them is relevant in order to use processed ingredients in food industry. A new utilization of tree nut flours could be obtained as a result of food processing but controlling their functional properties, as oil and water holding capacities, emulsifying, gelation or foaming properties, specially related to protein content and structure, as well as combination of several components as fat and carbohydrates (Aguilera et al., 2009). Evaluation of the changes of functional properties of tree nuts flours, after moist thermal treatment, is essential for a better understanding of their possible uses as a functional ingredient for food industry applications. In this study, the influence of moist thermal processing, without and with application of pressure (boiling and autoclave), on total protein content, phenolic compounds (anthocyanins, flavonols, tartaric esters and total phenols) content and antioxidant activity of pistachio, cashew and chestnut flours has been evaluated. The impact of boiling and autoclaving on the functional properties of pistachio, cashew and chestnut flours has also been analysed.

2. Materials and methods

2.1 Samples

Pistachio nuts (*Pistacia vera* L. var. Kerman) were provided by IRTA (Institut de Recerca I Tecnologia Agroalimentaries; Mas de Bover, Tarragona, Spain). The commercial unprocessed cashew (*Anacardium occidentale* L. type 320) and chestnut (*Castanea sativa* Mill. var. Miquelenca) were purchased to Productos Manzanares S.L (Albacete, Spain) and Cooperativa Secallona (Viladrau, Gerona, Spain), respectively.

2.2 Thermal and pressure processing

One hundred grams of nuts from pistachio var. Kerman, cashew type 320 and chestnut var. Miquelenca, were boiled in distilled water (1:5 w/v) for 30 and 60 minutes or autoclaved in distilled water (1:5 w/v) at two different temperature and pressure conditions (121°C/1.18 atm or 138°C/2.56 atm) for 15 and 30 min (Figure 1). Unprocessed (control) and processed nuts were freeze-dried (Telstar Cryodos, Terrasa, Spain), ground using a kitchen robot (Thermomix 31-1, Vorwerk Elektrowerke, GmbH & Co, KG, Wüppertal, Germany) and defatted with n-hexane (34 mL g^{-1} of flour) except chestnut flour which was not necessary to defat due to its low fat content (Borges et al., 2008). All samples were milled with a sieve of 1 mm (Tecator, Cyclotec 1093, Höganäs, Sweden).

2.3 Protein content and electrophoretic pattern

Total protein content of defatted flours from pistachio, cashew and chestnut unprocessed (control) and processed samples was determined in duplicate by Dumas method (LECO® analysis) using 5.3 as nitrogen-to-protein conversion factor (AOAC, 2003). Protein electrophoretic pattern from unprocessed (control) and processed pistachio, cashew and chestnut flours solubilized in SDS buffer was analysed by SDS-PAGE (Sanchiz et al., 2018).

20 µg of protein were loaded in a 4-20% Tris/Glycine SDS precast gels (Bio-Rad, Hercules, CA, USA), electrophoresed using a Mini-Protean 3-Cell (Bio-Rad, Hercules, CA, USA) and then visualized with Bio-Safe Coomassie G-250 (Bio-Rad, Hercules, CA, USA). The Coomassie-stained gels were scanned and the molecular weight of the bands was assessed using Quantity One software (Bio-Rad) and the Precision Plus SDS-PAGE protein mixture (Bio-Rad, Hercules, CA, USA) as standard.

2.4 Phenolic compounds

Flours from unprocessed (control) and processed nuts were extracted in duplicate with methanol-HCl (1‰): water (80:20 v/v) as described previously by Dueñas et al.(2006) with minor modifications. Briefly, 5 grams of sample flour were homogenized for 15 hours in extraction buffer (ratio 1:10 w/v) at orbital shaking at room temperature (RT). Supernatant was recovered after centrifugation at 4°C during 20 min at 23300 *g* and re-extracted as described above. Combined supernatants were evaporated to 5 mL under vacuum, using a rotary evaporator (Büchi rotary evaporator R-205, Büchi Labortechnik AG, Flawil, Switzerland) and then used for quantification of the total phenolic compounds (Oomah, Cardador-Martínez, & Loarca-Piña, 2005). Figure 1 shows the schematic representation of the sample processing and phenolic extraction. The absorbance of the methanolic extracts was monitored at 520, 360, 320 and 280 nm (Beckman spectrophotometer DU-7, Brea, CA, USA) using cyanidin-3-glucoside (0–20 µg mL^{-1}), quercetin (0–80 µg mL^{-1}), caffeic acid (0–30 µg mL^{-1}) and (+)-catechin (0–200 µg mL^{-1}) as standards for anthocyanins, flavonols, tartaric esters and total phenols, respectively.

2.5 Antioxidant Activity

Methanolic extracts from unprocessed (control), boiled for 60 min and autoclaved at 121°C and 138°C for 30 min samples from pistachio, cashew and chestnut were selected and used to

determine the antioxidant activity by the dimethyl-ρ-phenylenediamine (DMPD) assay, based on the reduction of N,N-dimethyl-ρ-phenylenediamine cation ($DMPD^{+}$) and following the manufacturer instructions (BioQuoChem, Llanera, Spain). Oxygen radical absorbing capacity (ORAC) was measured according to the method described by Dávalos et al.(2004) The standard curves for DMPD and ORAC assays were linear between 100-800 μM Trolox and the antioxidant activities were represented as μmol of Trolox equivalents g^{-1} of dry weight (DW) of sample.

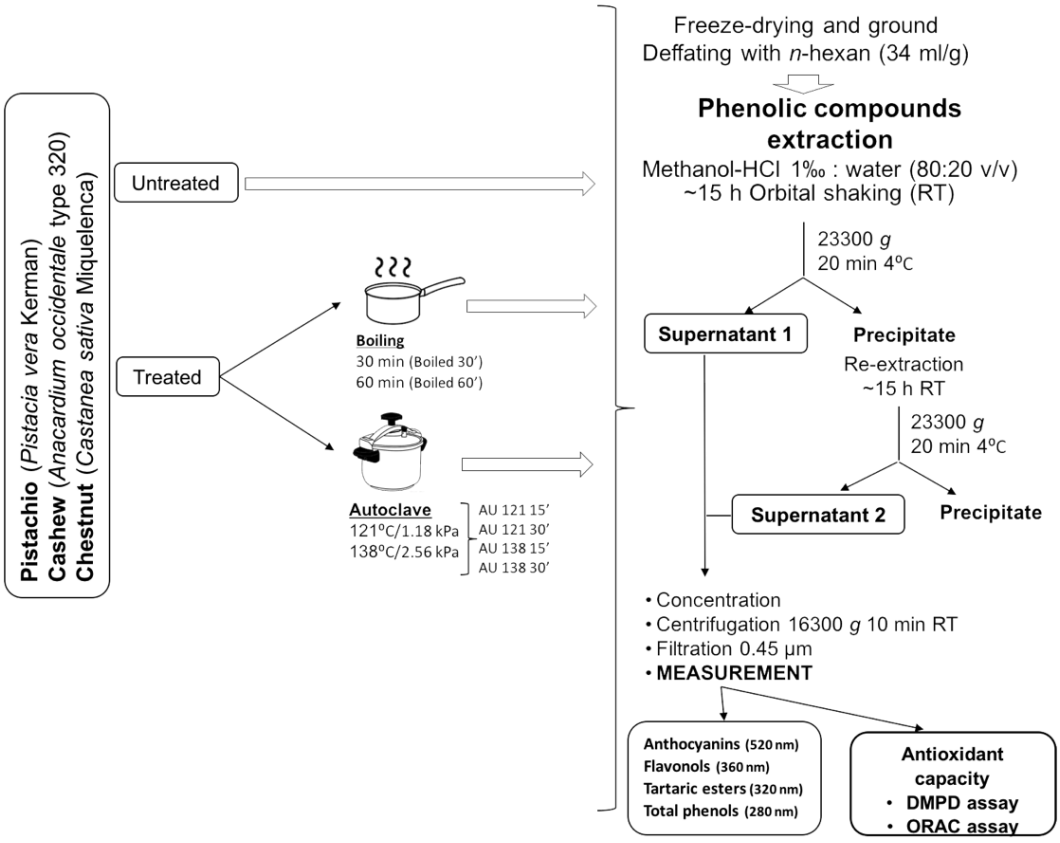

Figure 1. Schematic representation of the plant material, processing conditions and phenolic compounds extraction protocol.

2.6 Functional properties

Defatted flours from unprocessed (control), boiled for 60 min and autoclaved at 138°C 30 min pistachio, cashew and chestnut were selected to study their functional properties based on their

phenolic content and antioxidant activity. pH was measured according to official AOAC procedures (AOAC, 2003). Oil holding capacity (OHC) was measured following the method of Alobo et al. (2009) with minor modifications. After 30 min of continuous stirring, centrifugation was done at 2450 *g* for 30 min and OHC were expressed as mL of oil held g^{-1} of sample. Water holding capacity (WHC) were analysed according to the method described by Aguilera et al (2009) with minor modifications. One g of flour was stirred in plates with 10 mL of distilled water for 24 h at room temperature (RT). After centrifugation at 2450 *g* for 30 min, the supernatant volume was measured in a graduated cylinder. The WHC was expressed as mL of water held g^{-1} of sample (Aguilera et al., 2009). Water absorbing capacity (WAC) was determined following the methodology of Neto et al. (2001) with some variations. Briefly, 1 g of flour was mixed in 10 mL of distilled water for 1 h. Centrifugation was performed at 2750 *g* for 30 min at RT, and WAC was expressed as mL of water held g^{-1} of sample. The least gelation concentration (LGC) was determined by the method of Chau and Cheung (1998). Flour mixtures at 4, 8, 12, 14, 16, 18 and 20 % of flour (w/v) were prepared in 5 mL of distilled water, heated for 1 h in bath at 100 °C and cooled in ice for 1h. LGC was determined based on visual observation, when the sample did not fall after inversion of the tube (Siddiq, Nasir, Ravi, Dolan, & Butt, 2009). For emulsifying activity and its stability (EA and ES), 1 g of sample was mixed in 20 mL of water and 7 mL of oil at 13000 rpm for 2 min in an Ultraturrax homogenizer (IKA T-25). Centrifugation was performed at 2000 *g*, 30 min at 4 ºC and the emulsion volume was measured; EA was expressed as percentage of emulsified layer volume (Aguilera et al., 2009; Yasumatsu et al., 1972). For ES determination, tubes were transferred to a bath at 80 ºC for 30 minutes and the emulsified layer was measured. Finally, foaming capacity (FC) determination was based on the method of Bencini (1986). 0.5 g were mixed with 20 mL of distilled water in a homogenizer at highest speed for 5 min (Ultraturrax IKA T-10) and the foam volume was immediately measured (FC) and after 5 minutes to determine foam stability (FS).

2.7 Statistical analysis

One-way analysis of variance (ANOVA) was used to test the effect of processing on phenolic content, antioxidant activity and functional properties of the different species. Later on a Duncan multiple range test was applied to identify statistically significant contrasts. Differences were considered to be significant at $p<0.05$. To establish the association between the different phenolic compounds, both antioxidant activity assays as well as the phenolic compounds and antioxidant activity of flours Pearson's correlation coefficients were estimated. Fisher transformation tests were used to test if the estimated correlation coefficients were significantly different than zero (Table S1). All analyses were performed using Statgraphics Centurion X programme.

3. Results and discussion

3.1 Changes on total protein content and electrophoretic profile

Total protein content of unprocessed and processed samples showed slight changes (Figure 2A). All pistachio and cashew samples, showed higher protein content than chestnut ones. The chestnut total protein content was similar to that found in Portuguese chestnut varieties (Borges et al., 2008). In pistachio, autoclave treatments (especially AU 121 °C 30'and AU 138^{0}C 15 and 30') produced a significant increase compared to unprocessed control sample ($p<0.05$), whereas boiling has no effect on total protein content. This effect might be attributed to the loss of some soluble solid of the nuts after application of moist thermal treatments, which contributes to higher concentration of protein content, as explained by Pedrosa et al.(2015) in canned beans. In cashew and chestnut, processing did not affect the total protein content.

The SDS-PAGE profiles of total proteins from pistachio, cashew and chestnut defatted flours are presented in Figure 2B-D. Polypeptide bands range from 100 kDa to below 10 kDa in the unprocessed controls but the profile was specific for each tree nut. In boiled pistachio and cashew similar pattern was also visible compared to controls, for either 30 or 60 minutes, and

even in softly autoclaved samples (AU 121^{0}C 15'). In contrast, chestnut protein profile was more affected by these processing conditions, especially on higher molecular weight proteins. Autoclaving at 138^{0}C resulted in a drastic reduction of protein bands in the three nuts mainly in chestnut, probably due to higher resistance to hydrolysis of pistachio and cashew proteins compared to chestnut proteins (Cuadrado et al., 2018; De las Cuevas et al., 2018; Gimenez et al., 2018; Sanchiz et al., 2018).

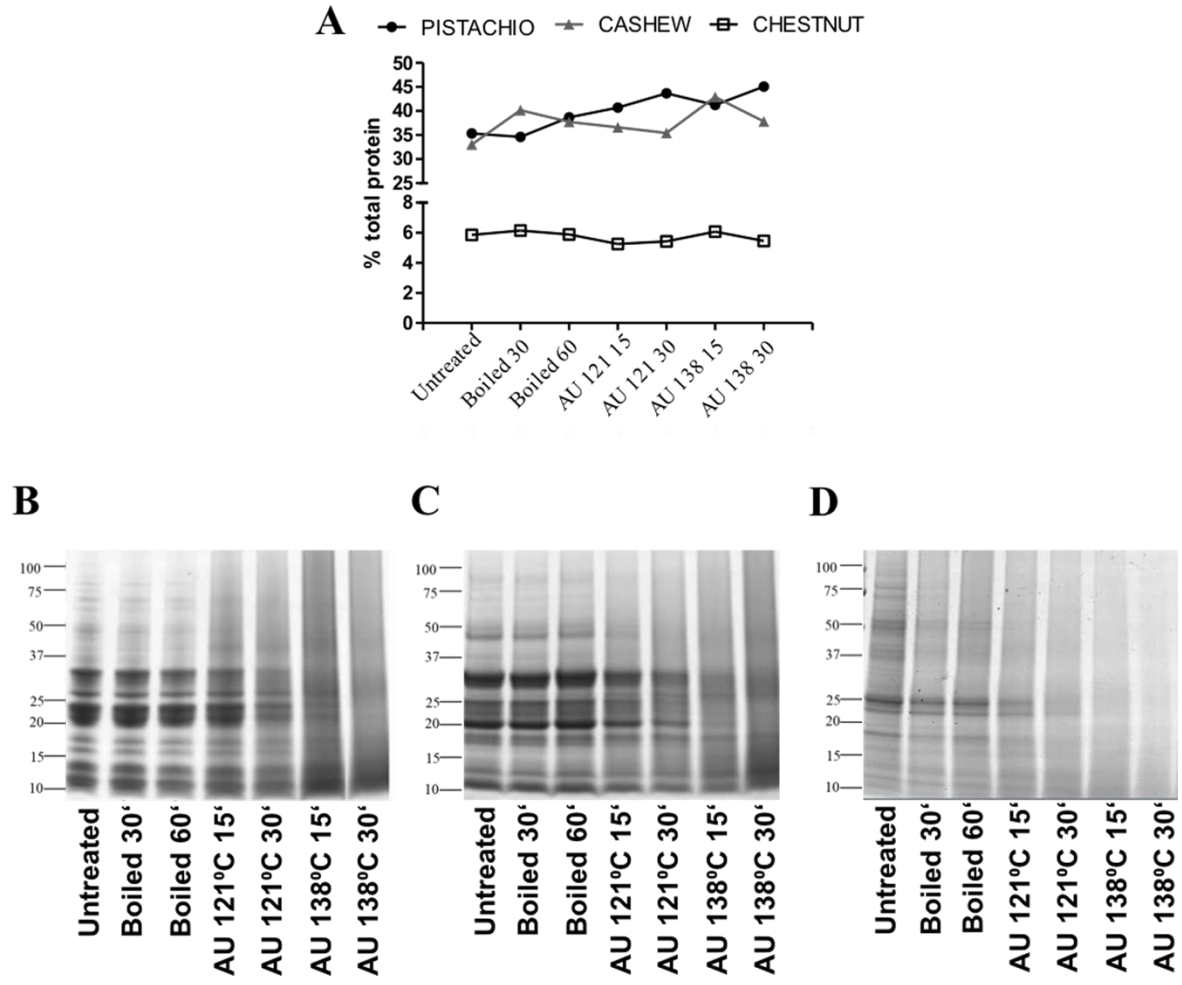

Figure 2. A) Total protein content of pistachio, cashew and chestnut defatted flours from processed and unprocessed (control) samples measured by LECO®. Electrophoretic SDS-PAGE pattern (4-20% of acrylamide) of flours solubilized in Laemmli sample buffer from unprocessed (control) and processed pistachio (B) and cashew (C) at 0.8 % w/v and D) chestnut at 4 % w/v. Molecular weight marker is Precision Plus (all-blue, BioRad).

3.2 Changes on phenolic compounds content

There are large differences in the colour of methanolic extracts among pistachio, cashew and chestnut (Figure S1), which were analysed to determine the phenolic compounds: anthocyanins, tartaric esters, flavonols and total phenols. The phenolic contents of pistachio, cashew and chestnut before and after processing are summarized in Table 1. Unprocessed pistachio showed higher quantity of total phenols compared to unprocessed cashew and chestnut (Table 1). Pistachio var. Kerman presented a cream-colored extract and total phenol content was not significantly different to other varieties, such as Mateur and Aegina (data not shown). Cashew type 320 and chestnut var. Miquelenca showed a lower amount of anthocyanins and total phenols being also less coloured samples (Figure S1).

Boiling for 30 and 60 minutes produced an important decrease in the quantity of total phenols, flavonols and tartaric esters in pistachio and cashew compared to unprocessed controls (Table 1). These losses can be attributed to the leaching of some phenols to the boiling water due to the softening of cells walls of nuts during processing as well as the different thermal stability of each particular phenol compound. Canning of beans produced a decrease in total phenolic content as a consequence of the industrial process (Pedrosa et al., 2015). Boiling treatment (for 40 minutes) provoked a loss of total phenols and flavonols content in several varieties of beans compared to unprocessed samples (Siah, Wood, Agboola, Konczak, & Blanchard, 2014). In contrast, boiling treatments leaded no significant reduction on the total phenol and flavonols content in chestnut (Table 1). Chestnut shows some important differences compared to other tree nuts as higher starch content and different nutrient profile (carbohydrates, fat, water content and protein quantity) (Cruz, Abraão, Lemos, & Nunes, 2013; Ros, 2010). The interactions between phenols and these nutrients might contribute to the different response from chestnut to boiling treatments, compared to pistachio and cashew.

Table 1. Determination of phenolic compounds (anthocyanins, flavonols, tartaric esters and total phenols) content of unprocessed and processed pistachio, cashew and chestnut flours. Mean (n=4) ± SE.

Samples	Anthocianins (mg cianidin/g)	Flavonols (mg quercetin/g)	Tartaric esters (mg cafeic acid/g)	Total phenols (mg catequin/g)
Pistachio				
Untreated	0.019 ± 0.001 ^{a}	0.278 ± 0.027 ^{d}	0.297 ± 0.018 ^{c}	9.404 ± 0.461 ^{d}
Boiled 30'	0.006 ± 0.001 ^{b}	0.180 ± 0.003 ^{bc}	0.216 ± 0.004 ^{a}	5.052 ± 0.357 ^{a}
Boiled 60'	0.019 ± 0.005 ^{a}	0.191 ± 0.006 ^{ab}	0.221 ± 0.004 ^{a}	5.730 ± 0.111 ^{ab}
AU 121 15	0.007 ± 0.001 ^{b}	0.200 ± 0.027 ^{c}	0.225 ± 0.029 ^{ab}	5.529 ± 0.816 ^{ab}
AU 121 30	0.015 ± 0.002 ^{a}	0.216 ± 0.009 ^{a}	0.245 ± 0.009 ^{b}	6.004 ± 0.201 ^{b}
AU 138 15	0.015 ± 0.005 ^{a}	0.275 ± 0.012 ^{d}	0.336 ± 0.015 ^{d}	7.8713 ± 0.962 ^{c}
AU 138 30	0.016 ± 0.002 ^{a}	0.322 ± 0.028 ^{e}	0.353 ± 0.009 ^{d}	8.797 ± 0.720 ^{d}
Cashew				
Untreated	0.005 ± 0.002 ^{ab}	0.033 ± 0.002 ^{d}	0.132 ± 0.008 ^{a}	4.727 ± 0.513^{a}
Boiled 30'	0.015 ± 0.003 ^{d}	0.019 ± 0.001 ^{bc}	0.095 ± 0.003 ^{b}	3.174 ± 0.133 ^{b}
Boiled 60'	0.035 ± 0.003 ^{e}	0.017 ± 0.002 ^{ab}	0.079 ± 0.005 ^{c}	2.848 ± 0.271 ^{b}
AU 121 15	0.009 ± 0.002 ^{bc}	0.022 ± 0.007 ^{bc}	0.073 ± 0.009 ^{c}	2.431 ± 0.313 ^{c}
AU 121 30	0.003 ± 0.001 ^{a}	0.012 ± 0.001 ^{a}	0.061 ± 0.003 ^{d}	1.901 ± 0.072 ^{d}
AU 138 15	0.009 ± 0.003 ^{bc}	0.032 ± 0.003 ^{d}	0.089 ± 0.005 ^{b}	2.366 ± 0.106 ^{c}
AU 138 30	0.009 ± 0.004 ^{c}	0.054 ± 0.002 ^{e}	0.128 ± 0.004 ^{a}	2.945 ± 0.010 ^{b}
Chestnut				
Untreated	0.001 ± 0.001 ^{a}	0.053 ± 0.000 ^{a}	0.175 ± 0.004 ^{a}	3.184 ± 0.034 ^{a}
Boiled 30'	0.003 ± 0.001 ^{a}	0.045 ± 0.004 ^{a}	0.149 ± 0.013 ^{a}	3.244 ± 0.307 ^{a}
Boiled 60'	0.003 ± 0.001 ^{a}	0.044 ± 0.002 ^{a}	0.128 ± 0.004 ^{a}	2.846 ± 0.149 ^{a}
AU 121 15	0.004 ± 0.001 ^{ab}	0.074 ± 0.004 ^{ab}	0.179 ± 0.012 ^{a}	3.718 ± 0.229 ^{ab}
AU 121 30	0.006 ± 0.001 ^{b}	0.105 ± 0.002 ^{b}	0.258 ± 0.010 ^{b}	4.470 ± 0.025 ^{b}
AU 138 15	0.010 ± 0.001 ^{c}	0.1930± 0.010 ^{c}	0.368 ± 0.004 ^{c}	6.518 ± 0.072 ^{c}
AU 138 30	0.019 ± 0.001 ^{d}	0.340 ± 0.006 ^{d}	0.632 ± 0.007 ^{d}	9.981 ± 0.128 d

Values are means ± standard error (n=4); Means values in the same column followed by a different superscript are significantly different ($p<0.05$).

Autoclaved pistachios at 138 °C (2.56 atm) for 30 min showed similar amount of total phenols than unprocessed, or even slightly higher values, for flavonols and tartaric esters (Table 1). The same treatment in cashew did not reduce phenolic compounds compared to other treatments (boiling or autoclave at 121ºC), although the lowest amount of phenolic compounds was found in cashew after autoclave at 121 °C for 30 minutes (Table 1). When autoclave was applied at the harshest conditions, the content of total phenols in chestnut was the maximum value detected, close to 10 mg catechin g^{-1} of sample, which is more than 3-fold increase relative to unprocessed chestnuts (Table 1). The results were similar for the rest of phenolic components; autoclave 138˚C for 30 minutes provoked an increase of at least 3.6-fold in the case of tartaric esters and up to 14-fold in anthocyanins compared to control. Other treatments, as roasting, significantly increased total phenolic content on nuts, compared to unprocessed samples

(Chandrasekara & Shahidi, 2011b; Vinson & Cai, 2012). Akbari et al.(2017) applied gamma radiation to three different pistachio varieties, obtaining increased values of total phenols and anthocyanins in a dose-dependent mode. These authors explained that gamma radiation might alter the enzymatic activity from phenylpropanoid pathway, increasing the quantity of phenolic compounds. However, autoclave treatment caused losses in phenolic contents, as well as antioxidant activity in *Vicia faba* and *Phaseolus vulgaris* that are attributed to chemical transformation, decomposition of phenolics, and formation of phenolic-protein complex under thermal and pressure conditions (Oomah et al., 2005; Siah et al., 2014). The increase obtained here in the content of specific phenolic compounds of chestnut after autoclave at harshest conditions can be due to higher extraction yields, the formation of Maillard reaction products as well as the possible release of some bound phenolic compounds due to the processing conditions applied (Chandrasekara & Shahidi, 2011a, 2011b). This might also contribute to the observed change of extract colour, darker than unprocessed and boiled chestnut (Figure 1S). The analysis of association between phenolic compounds indicated a high positive correlation was found between total phenols and flavonols ($R=0.957$, $p<0.0001$), total phenols and tartaric esters ($R=0.867$, $p<0.0001$) and between flavonols and tartaric esters ($R=0.873$, $p<0.0001$) (Table S1).

3.3 Changes on antioxidant activity

According phenolic compounds data, unprocessed, boiled 60 min, AU 121 °C and AU 138 °C for 30 min samples were selected, in pistachio, cashew and chestnut, to determine the influence of processing on the antioxidant activity by DMPD and ORAC assays. The results are represented in Figure 3 as μmol Trolox g^{-1} of sample. The antioxidant capacity of pistachio and chestnut was not significantly affected by boiling neither autoclave processing, measured by DMPD assay. In contrast, both treatments diminished antioxidant capacity in cashew when

were determined by DMPD assay. When the samples were measured by ORAC assay, both pistachio and cashew nuts autoclaved at 121°C showed the lowest values of antioxidant activity, which also contained lower amount of total phenols in both nuts (Table 1). In chestnut, boiling showed lower total phenol content among treatments and revealed lower antioxidant activity by ORAC assay. Autoclave at 138°C, 30 min did not reduce antioxidant activities of pistachio, cashew and chestnut compared to other processed samples, reaching even higher values in chestnut (Figure 3).

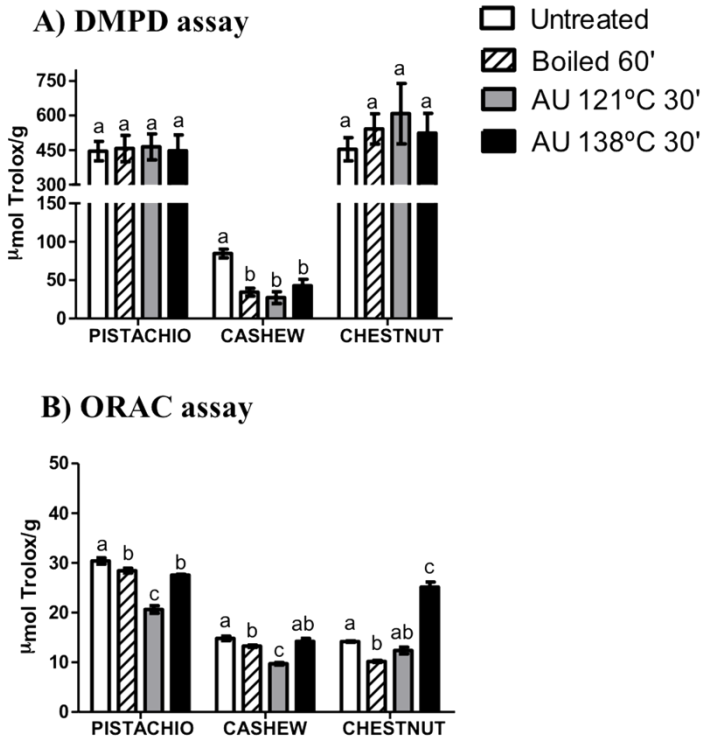

Figure 3. Determination of antioxidant activity by DMPD (A) and ORAC (B) assays from unprocessed (control) and processed pistachio, chestnut and cashew flours. Different letterS indicate significant differences by Duncan test ($p<0.05$).

Significant linear correlation between total phenols and antioxidant activities, determined by DMPD as well as ORAC assays, was found (Table S1). The same association analysis also revealed positive correlation between both assays (Table S1). Autoclave treatment (121°C, 10 min) applied to different *P. vulgaris* cultivars produced an increase of antioxidant activity in Peruvian cultivars and did not change in the Brazilian ones, despite the total phenolic content

decreased in all bean cultivars (Ranilla, Kwon, Genovese, Lajolo, & Shetty, 2010). Aguilera et al. (2013) demonstrate that germination is a very effective natural process to increase bioactive phenolic compounds and antioxidant activity in non-conventional legumes being both significantly correlated. In contrast, roasting had a negative effect on individual phenolics but not on the total phenolic content and antioxidant potential of hazelnut kernels (Schmitzer, Slatnar, Veberic, Stampar, & Solar, 2011). Gamma irradiation produced higher phenolic, anthocyanin and antioxidant activity in different pistachio nuts (Akbari et al., 2017). In defatted sesame meals, roasting treatment at different temperatures and times gradually increased antioxidant activity, as well as phenolic composition (Jeong et al., 2004), similarly to our findings in processed chestnut (Table 1). (Chandrasekara & Shahidi (2011b) observed that roasting at 130°C for 33 minutes produced the highest antioxidant activity in cashew nuts, determined by several methods. Moist thermal treatments applied in this study based on boiling and autoclave, especially at harsh conditions, had no strong effect on reduction of antioxidant activity determined by DMPD and ORAC assays.

3.4 Changes on functional properties of nut flours

The behaviour of food ingredients depends on their physical and chemical properties, which can be affected by processing and storage. Unprocessed, boiled (60 min) and autoclaved (138°C for 30 min) defatted flours were selected for determination of some functional properties of pistachio, cashew and chestnut, which are presented in Table 2. It is relevant to measure the pH values of the flours in water because many others functional properties can be affected by pH changes. Similar pH values have been obtained among samples, being slightly more acid in chestnut than in pistachio and cashew. Treatment by boiling produced a slight increase in pH of all nuts (Table 2). In relation to OHC, it was higher in cashew than in pistachio and chestnut. Thermal treatments increased OHC, especially after autoclave processing, compared to control

pistachio and cashew. Better ability to absorb oil by processed flours might be related to conformational changes on proteins, due to thermal treatment, i.e., permitting exposure of more hydrophobic/hydrophilic parts bound to oil units (Ling, Zhang, Li, & Wang, 2016). OHC values in chestnut were around 3.2 mLg^{-1} despite the applied treatment (Table 2). Similarly, some legume flours, did not show different OHC values between control samples and cooked and dehydrated seeds (Aguilera et al., 2009; Aguilera, Estrella, Benitez, Esteban, & Martín-Cabrejas, 2011). The processed samples may be better-suited flours for confectionery applications requiring oil emulsification.

Different structure or conformational characteristics of proteins after thermal treatment, both boiling and autoclave, might be responsible of observed augment on WHC. Protein content is related to WHC, which is higher in pistachio and cashew than in chestnut (Figure 2). Other compounds from seeds and nuts can also be taking a role in techno-functional properties of defatted flours as fibre and starch (Aguilera et al., 2009), considerably high in chestnut (Cruz et al., 2013; Rodríguez-Miranda et al., 2011; Tosh & Yada, 2010). Dissociation of proteins can be responsible of higher number of water molecules bound to polar rests from polypeptide chains (Ling et al., 2016; Neto et al., 2001). Studies performed with a variety of legumes found similar results when thermal treatment was applied to seeds, as cooked vitabosa, chickpea and lentils (Aguilera et al., 2009; Chaparro, Gil, & Aristizábal, 2011).

Table 2. Functional properties of unprocessed (control) and processed tree nut flours. *pH, OHC (oil holding capacity), WHC (water holding capacity), WAC (water absorption capacity), LGC (least gelation capacity), emulsifying* and *foaming* capacities and stabilities are included.

	pH	OHC (ml/g)	WHC (ml/g)	WAC (ml/g)	LGC (%)	Emulsifying (%)		Foaming (%)	
Pistachio						*Capacity*	*Stability*[1]	*Capacity*	*Stability*[2]
Untreated	5.99	3.10 ± 0.30[a]	3.50 ± 0.00[a]	2.25 ± 0.05[a]	8	25.2 ± 0.50[a]	15.9 ± 0.50[a]*	47.4 ± 0.00[a]	45.7 ± 0.00[a]
Boiled 60'	6.26	3.65 ± 0.15[ab]	4.10 ± 0.10[b]	2.80 ± 0.00[b]	8	21.6 ± 1.00[b]	19.5 ± 1.03[b]	23.1 ± 0.00[b]	21.2 ±1.90[b]
AU 138°C 30'	5.71	4.40 ± 0.20[b]	4.60 ± 0.20[b]	2.70 ± 0.00[b]	8	4.1 ± 0.00[c]	4.1 ± 0.00[c]	24.2 ± 1.60[b]	4.80 ± 0.90[c]*
Cashew									
Untreated	6.17	4.30 ± 0.10[a]	2.75 ± 0.25[a]	1.00 ± 0.00[a]	8	28.8 ± 0.00[a]	28.8 ± 0.00[a]	23.1 ± 0.00[a]	9.1 ± 0.00[a]*
Boiled 60'	6.20	5.10 ± 0.10[b]	3.85 ± 0.05[b]	3.05 ± 0.05[b]	8	12.1 ± 0.20[b]	12.1 ± 0.20[b]	12.3 ± 2.20[b]	9.1 ± 0.00[a]*
AU 138°C 30'	5.98	5.40 ± 0.00[b]	4.35 ± 0.15[b]	4.20 ± 0.00[c]	12	10.5 ± 0.00[c]	10.5 ± 0.00[c]	23.1 ± 0.00[a]	9.1 ± 0.00[a]*
Chestnut									
Untreated	5.63	3.15 ± 0.25[a]	2.50 ± 0.00[a]	1.05 ± 0.05[a]	8	27.8 ± 1.00[a]	20.6 ± 0.00[a]*	35.4 ± 2.1	25.8 ± 2.70*
Boiled 60'	5.78	3.20 ± 0.00[a]	3.75 ± 0.15[b]	2.60 ± 0.00[b]	18	03.1 ± 1.03[b]	03.1 ± 1.03[b]	ND	ND
AU 138°C 30'	5.22	3.30 ± 0.10[a]	4.40 ± 0.00[c]	3.10 ± 0.00[c]	16	04.1 ± 0.00[b]	04.1 ± 0.00[c]	ND	ND

Values are means ± standard error (n=4); Means values in the same column followed by a different superscript are significantly different ($p<0.05$).
[1]after 30 minutes at 80°C
[2]after 5 min at RT
*Indicates significant differences between capacity and stability within the same sample ($p<0.05$).
ND = Not detected

WAC was higher in pistachio flours than in cashew and chestnut. In all cases, boiling and autoclave treatment increased this property. Neto et al. (2001) demonstrated that autoclave treatment on cashew protein isolate also improved WAC compared to control. Gelation capacity is mainly a feature from proteins and polysaccharides and it depends on protein-protein or protein-polysaccharide interactions. Gelling power is interesting in food industry for dairy products, jellies and desserts (Siddiq et al., 2009). In our samples, complete gelation was observed at 8% (LGC) or higher concentration of raw flours (Table 2). In processed flours, LGC was not affected in pistachio but highly in chestnut. Cashew maintained LGC in boiled samples and autoclave treatment increased this capacity to 12% of flour. The aggregation of denatured proteins beside the protein structure changes due to the processing might lead to a reduction of gelation capacities (Aguilera et al., 2011). Flours from unprocessed and boiled pistachio revealed similar EC, as previously has been described in roasted pistachio flour (Ling et al., 2016). Thermal treatment combined with pressure affected EC in this nut (Table 2). Differently, both thermal processes produced a reduction in emulsifying properties of cashew and especially chestnut, as observed by other authors in pulses' flours (Aguilera et al., 2009, 2011). In cashew, emulsifying stability was 100% either in control and processed samples, as described in cashew protein isolate from roasted nuts (Neto et al., 2001). Control pistachio, followed by chestnut, showed the highest foaming capacities (Table 2). Moist thermal treatments caused a reduction, or even an elimination, of FC probably because loss or change of structural and conformational properties of proteins, which hampered foam formation. Roasting treatment also triggered a reduction on FC of partially defatted and totally defatted pistachio flours (Ling et al., 2016). Emulsifying and foaming properties are interesting for preparation of some sauces, ice cream or cakes, as well as soups and some spreadable products.

4. Conclusions

Tree nuts are a rich source of proteins, carbohydrates and polyphenols, with probed healthy benefits. Our findings indicate that boiling treatment reduced most of phenolic compounds in pistachio and cashew, but had no strong effect on chestnut, compared to control samples. In contrast, treatments based on heat and pressure increased gradually the total phenol content, reaching similar levels to control samples or even higher in chestnut. Antioxidant activity measured by DMPD and ORAC assays in processed pistachio, cashew and chestnut was slightly affected after boiling and maintained or even increased after autoclave at more drastic conditions. OHC, WHC and WAC increased after moist thermal processing in pistachio and cashew. Emulsifying and foaming capacities were altered by thermal treatment. LGC in processed chestnut flour was mainly influenced by thermal treatment. Consequently, pistachio, cashew and chestnut flours with reduced allergenic properties and maintaining their phenolic content and antioxidant activity can be obtained by application of technological processing combining heat and pressure. Therefore, these processed tree nuts could be considered safer for allergic subjects and as healthy and suitable for food industry as the unprocessed ones.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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Appendix 3. Supplementary Material

Table S1. Pearson's correlation coefficients between the different phenolic compounds (anthocyanins, flavonols, tartaric esters and total phenols), both antioxidant activity assays (DMPD and ORAC) as well as the phenolic compounds and antioxidant activity of flours were estimated. Fisher transformation tests were used to test if the estimated correlation coefficients were significantly different (*) ($p<0.05$)

Comparison	Pearson's correlation coefficient	p value
Total phenol-Flavonol	0,9568*	<0,0001
Total phenol-Anthocyanin	0,3866*	0,0066
Total phenol-Tartaric ester	0,8668*	<0,0001
Flavonol-Anthocyanin	0,3786*	0,0079
Flavonol-Tartaric ester	0,8732*	<0,0001
Anthocyanins-Tartaric ester	0,2468	0,0916
ORAC-DMPD	0,3506*	0,0146
ORAC-Total phenol	0,8738*	<0,0001
ORAC-Flavonol	0,8814*	<0,0001
ORAC-Anthocyanin	0,4719*	0,0007
ORAC-Tartaric ester	0,6501*	<0,0001
DMPD-Total phenol	0,434*	0,0021
DMPD-Flavonol	0,497*	0,0003
DMPD-Anthocyanin	-0,13	0,3785
DMPD-Tartaric ester	0,5295*	0,0001

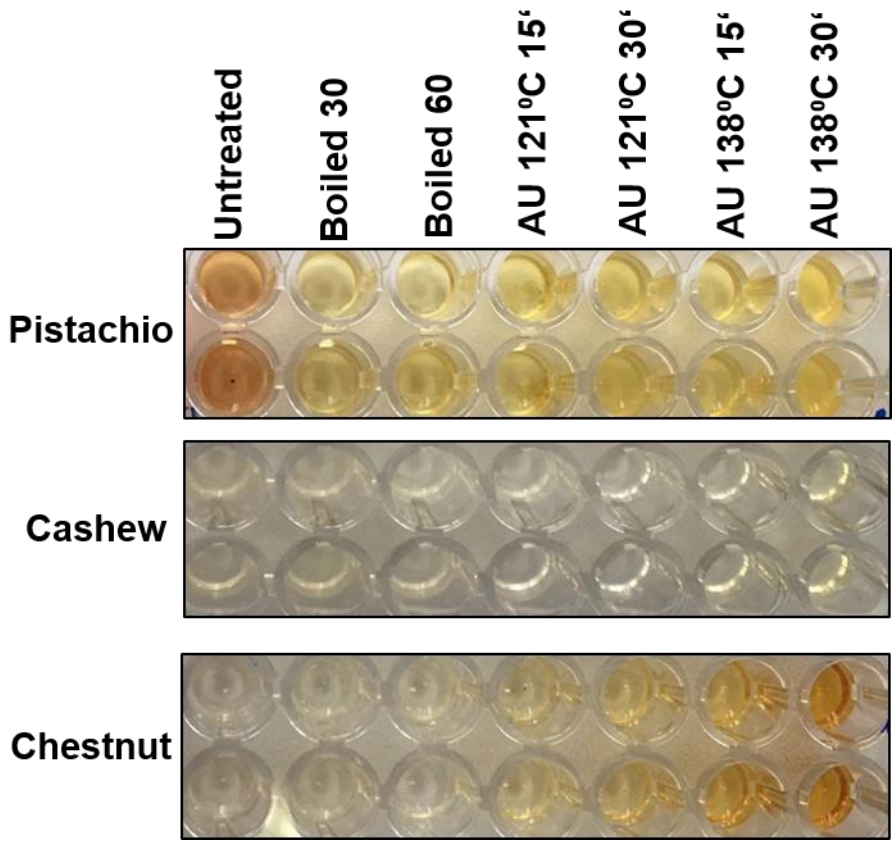

Figure 1 Supplementary material. Appearance of methanolic extracts from pistachio (A=Aegina, L= Larnaka, M= Mateur, S= Sirora, K= Kerman) and chestnut varieties (C= Cella Ampla, PA= Pere Andreu, P= Primerenc, T = Tarda, M= Miquelenca) and from untreated and treated pistachio, chestnut and cashew samples (1= untreated, 2= Boiled 30, 3= Boiled 60, 4= AU 121°C 15´, 5= AU 121°C 30´, 6= AU 138°C 15´, 7= AU 138°C 30´).

BLOQUE II

Detección de frutos secos en muestras tratadas por PCR en tiempo real

Artículo 4

DETECCIÓN POR PCR EN TIEMPO REAL DE SECUENCIAS CODIFICANTES DE ALÉRGENOS DE NUEZ EN ALIMENTOS PROCESADOS

DETECTION BY REAL TIME PCR OF WALNUT ALLERGEN CODING SEQUENCES IN PROCESSED FOODS

Se ha puesto a punto y validado un método cuantitativo de PCR en tiempo real, empleando parejas de cebadores diseñadas a partir de las secuencias codificantes de los alérgenos Jug r 1, Jug r 3, and Jug r 4. Se ha evaluado la especificidad, sensibilidad y aplicabilidad del ensayo. El mejor método de extracción de ADN de nuez fue el basado en CTAB-fenol-cloroformo. La RT-PCR permitió la amplificación específica y precisa de las secuencias diana, con un límite de detección de 2.5 pg de ADN de nuez. La sensibilidad y fiabilidad del método se ha confirmado usando mezclas binarias (spiked), y los cebadores Jug r 3 detectaron hasta 100 mg/kg de nuez cruda (LOD 0.01%, LOQ 0.05%). El tratamiento térmico combinado con presión (autoclave) redujo el rendimiento y la amplificación del ADN de nuez (integridad y calidad). Las altas presiones hidrostáticas (HHP) no afectaron a la amplificación del ADN de nuez. Este método de PCR en tiempo real mostró mayor sensibilidad y precisión para detectar trazas de nuez en alimentos comerciales que los ensayos ELISA.

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Detection by real time PCR of walnut allergen coding sequences in processed foods

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ABSTRACT

A quantitative real-time PCR (RT-PCR) method, employing novel primer sets designed on Jug r 1, Jug r 3, and Jug r 4 allergen-coding sequences, was set up and validated. Its specificity, sensitivity, and applicability were evaluated. The DNA extraction method based on CTAB–phenol–chloroform was best for walnut. RT-PCR allowed a specific and accurate amplification of allergen sequence, and the limit of detection was 2.5 pg of walnut DNA. The method sensitivity and robustness were confirmed with spiked samples, and Jug r 3 primers detected up to 100 mg/kg of raw walnut (LOD 0.01%, LOQ 0.05%). Thermal treatment combined with pressure (autoclaving) reduced yield and amplification (integrity and quality) of walnut DNA. High hydrostatic pressure (HHP) did not produce any effect on the walnut DNA amplification. This RT-PCR method showed greater sensitivity and reliability in the detection of walnut traces in commercial foodstuffs compared with ELISA assays.

1. Introduction

There is interest in walnuts (*Juglans regia* L.) because their consumption has been demonstrated to be a healthy dietary habit. Regular consumption of walnuts is associated with lower cholesterol, which reduces the risk of coronary heart disease (Ros, 2010). After peanut, walnut and other tree nuts represents the foods most commonly involved in severe allergic reactions (Sicherer, Furlong, Muñoz-Furlong, Burks, & Sampson, 2001). The importance of walnut- and other nuts allergies is related to not only to the severity of the reactions, but also prevalence in the general population (from 0.2% to 0.7%) (Emmett, Angus, Fry, & Lee, 1999). The true prevalence of food allergies is difficult to establish because they depends on many factors and can vary across different geographical areas. In Europe, allergy to tree nuts is relatively common, with hazelnut the major contributor, whereas in the USA walnut, almond and cashew are most frequently involved in allergic reactions (Ortolani et al., 2000; Sicherer, Muñoz-Furlong, & Sampson, 2003).

Four walnut proteins are identified as allergens in the allergen WHO-IUIS list (Allergen, 2013): Jug r 1 (2S albumin), Jug r 2 (7S globulin or vicilin), Jug r 3 (lipid transfer protein, LTP) and Jug r 4 (11S globulin or legumin). Jug r 5 (profilin) has also been described as a walnut allergen, but has not yet been included in the list. Currently, the only 'treatment' for allergic patients is the complete elimination of foods triggering their allergy from the diet. Thus, allergic patients have to rely on labelling information, especially for processed foods (Niemann, Taylor, & Hefle, 2009). In Europe, walnuts are included on the list of allergens that must always be declared on food labels (Regulation (EU) No 1169/2011). However, walnuts can be found as a hidden allergen due to cross-contamination during processing. The need for adequate methodologies to detect allergenic ingredients has prompted the development of numerous techniques. Nevertheless, the lack of available reference materials, and the absence of official methods for allergen detection and quantification, represent a major difficulty in the management of food allergens.

Abbreviations: Ct, cycle threshold; CTAB, hexadecyltrimethylammonium bromide; LTP, lipid transfer protein; HHP, high hydrostatic pressure; NTC, non template control; RT, real time.

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Enzyme-linked immunosorbent assay (ELISA) is the most frequently used method for the detection of allergens in complex food matrices (Costa, Carrapatoso, Oliveira, & Mafra, 2014). Because thermal/pressure treatments may alter the protein and its extraction characteristics, the reliability of these detection techniques in processed foods can be reduced (Sathe & Sharma, 2009). DNA-based methods, in which the target molecules are specific DNA sequences amplified by the polymerase chain reaction (PCR), are a good alternative for the detection of allergenic ingredients because DNA from food matrices is less affected by thermal treatment and other processing conditions (Poms, Klein, & Anklam, 2004). Quantitative RT-PCR (RT-PCR), using fluorescence probes, has been the most widely applied PCR strategy to detect food allergens. The DNA target sequence used has to be a species-specific region of the allergenic food or a gene encoding of an allergenic protein (Scaravelli, Brohee, Marchelli, & van Hengel, 2009). Conventional (Yano et al., 2007) and RT-PCR have been used to detect walnut in food samples using different dye methods and target sequences, such as Jug r 2 (Brezná, Piknová, & Kuchta, 2009; Wang et al., 2009) and Jug r 3 (Costa, Oliveira, & Mafra, 2013).

Walnuts are mainly ingredients of foodstuffs such as bakery products or confectionery. Usually, consumers are exposed to ingredients in complex matrices that are subjected to several processing methods (e.g. physical, chemical and enzymatic) to improve their quality, shelf-life, safety, suitability for specific product applications, etc. Recently, HHP has been more widely applied as a promising technology to modify functional properties of proteins. It has been reported that the primary structure of proteins remains intact during processing while secondary, tertiary, and quaternary structures are affected to different degrees (Chapleau, Mangavel, Compoint, & Lamballerie-Anton, 2004). This processing method has, therefore, the potential to modify food proteins, which could alter food allergenicity, i.e. increasing or decreasing IgE reactivity (Burbano & Cuadrado, 2013; Cabanillas et al., 2012). To date, the effects of HHP on food allergenicity have been studied in beef (Yamamoto et al., 2010), apple (Husband et al., 2011; Johnson et al., 2010), celery (Husband et al., 2011) and peanut (Johnson et al., 2010). The allergenicity of these food was not modified by HHP, but an increase in soybean antigenicity has been reported (Peñas, Gomez, Frias, Baeza, & Vidal-Valverde, 2011). The effects of HPP techniques on tree nuts allergenicity were recently examined by our group. We found no significant effects of HHP on walnut allergenicity (Cabanillas et al., 2014) and differential solubility of hazelnut immunoreactive proteins (Prieto, Burbano et al., 2014a).

There are several reports where a negative effect on DNA detection after severe heat treatment has been described (Costa et al., 2013; Iniesto et al., 2013; Scaravelli et al., 2009). Detection of a DNA target has limitations. Firstly, the small amount of the target DNA in most food matrices requires a sensitive and specific methodology (Broeders, De Keersmaecker, & Roosens, 2012). Nevertheless, it is important to analyse the effects that food processing might exert on DNA molecules. Some processing methods might induce the fragmentation and/or degradation of genomic DNA (Ballari & Martin, 2013; Gryson, 2010;Prieto, Iniesto et al., 2014b). As far as we know, only a few studies have focused on the evaluation of food processing effects on DNA from tree nuts in general, and on walnuts in particular.

This aim of study was to set up and validate a SYBR Green RT-PCR method using specific primer pairs based on Jug r 1, Jug r 3, and Jug r 4 allergen-coding sequences to improve the sensitivity of RT-PCR techniques for detection of walnut traces in commercial food products. We also aimed to investigate the influence of high temperature and/or high pressure on the detection of walnut DNA targets in complex food matrices.

2. Materials and methods

2.1. Samples

All species of walnut studied are used as food ingredients and included the three main types of walnuts, "Persian walnut" or "European walnut" (*J. regia* L.), "Northern black walnut" (*Juglans nigra* L.), which is one of the main US species of black walnut, and "Butternut" (*Juglans cinerea* L.), which is from cold areas of North America and Canada (Duke, 1989). This study also included "Pecans" or "American walnut" (*Carya illinoinensis* (Wangenh.; K. Koch), (Madden, 1979). Samples were chosen from different origins for each species; three for *J. regia*, including the main walnut cultivar for fruit production 'Chandler', eight for *J. nigra*, one for *J. cinerea* and six for *C. illinoinensis*. All samples were obtained from IRTA's (Institut de Recerca i Tecnologia Agroalimentàries) Juglans Clonal bank (Germain, 2004). Other plants commonly used as food ingredients (almond, pistachio, cashew, hazelnut, peanut, rice, wheat, barley, and rye) were used in the specificity studies.

For the preparation of standards or spiked samples, binary mixtures containing 5, 10, 50, 100, 500, 1000, 5000, 10,000, 50,000 and 100,000 mg/kg (0.0005–10% w/w) defatted raw walnut "Chandler" flour in spelt (*Triticum spelta* L.) flour matrices were prepared to a final weight of 100 g. Commercial foodstuffs (chocolates, biscuits, snacks, etc) purchased from local food stores were used for analytical validation of the method. All samples were ground and homogenized using a kitchen robot (Thermomix 31-1, Vorwerk Elektrowerke, GmbH & Co. KG, Wüppertal, Germany).

2.2. Heat and HHP treatments

Walnuts (*J. regia* cv Chandler) were immersed in distilled water (1:5 w/v) and autoclaved using a Compact 40 Benchtop autoclave (Priorclave, London, UK) at 121 °C (120 kPa) and at 138 °C (260 kPa) for 15 and 30 min. Raw and autoclaved walnuts were ground and defatted with n-hexane (34 ml/g of flour) for 4 h and air-dried after removal of the n-hexane by filtration.

High pressure experiment conditions were carried out according to Omi, Kato, Ishida, Kato, and Matsuda (1996), Kato, Katayama, Matsubara, Omi, and Matsuda (2000).

Walnut defatted flours were dissolved in distilled water (1:4 w/v) 20 h before treatment and the suspensions subjected to HHP under 300, 400, 500 and 600 MPa for 15 min in multivessel high-pressure equipment (HHP, ACB, France) at 15 °C. Defatted flour from non-HHP-treated walnut, soaked for 20 h in distilled water, was used as the control for HPP-treated samples.

2.3. DNA isolation

DNA from raw walnuts was extracted using the CTAB/phenol/chloroform-based method previously described by Iniesto et al. (2013). DNA was cleaned up by passing it through a silica membrane in a spin column from Power Plant Pro DNA Isolation Kit (MoBio, CA, USA), and eluted in 50 μL H_2O Milli-Q.

The CTAB-based extraction was selected to obtain DNA from raw and processed walnuts (200 mg) as well as from spiked samples (100 mg) and complex food matrices (400 mg). The quantity and quality of DNA were determined with a NanoDrop ND-1000 Spectrophotometer (ThermoFisher, MA, USA) by measuring absorbance at 230, 260 and 280 nm. DNA quality was also evaluated on 0.8% TBE agarose gels.

2.4. Conventional PCR, cloning, sequencing and RT-PCR primer design

Oligo Primer Analysis Software was used to design the primer pairs for target coding sequences in three Jug r genes: Jug r 1

(GenBank accession No. U66866, Jug r 1 up GGCAGCATTTCAGGCAGT; Jug r 1 rp CCAGTTATCTTCCAGCGACAA), Jug r 3 (GenBank accession No. EU780670, Jug r 3 up CCAGTTGCAGAGGCGGTCATA; Jug r 3 rp AGGCCAAGCAGCAAGACCAGGG), Jug r 4 (GenBank accession No. AY692446, Jug r 4 up TGCCGTCGTTCGTCGTA; Jug r 4 rp GGCCTTCCACTCTCACTATGC).

These primers were used in conventional PCRs. These PCR reactions were carried out in 20 μL, containing 25 ng of DNA, 0.25 μM of each primer and 1× Taq PCR Master Mix (Qiagen, Germany). A SensoQuest LabCycler (Progen Scientific Ltd, Germany) was used with the following PCR program: initial denaturation step at 95 °C, 4 min, followed by 35 cycles of denaturation at 94 °C for 45 s; annealing at 60–64 °C for 1 min; elongation at 72 °C for 1 min; last step at 72 °C for 6 min.

PCR reactions were carried out using DNA from the three cultivars of walnut, and the amplicons with the expected size were cloned in *Escherichia coli* using the pGEM®-Tplasmid (*pGEM®-T Easy* Promega, Madison, WI, USA) as the vector. Recombinant plasmids were isolated using High Pure Plasmid Isolation Kit (Roche, Germany), and sequenced in a ABI PRISM 3700 sequencer (Applied Biosystems, CA, USA) from "Unidad de Genómica, Parque Científico de Madrid". After determination of the DNA sequences using Bio Edit (Carlsbad, CA, USA), a sequence comparison was carried out against those in the GenBank (NCBI) non-redundant nucleotide (nr/nt) databases using BLAST. ClustalW algorithm was used to perform the alignments between sequences of the same gene obtained from the three walnut cultivars The conserved sequences of Jug r 1, 3, and 4 were used to design the specific primer pairs for RT-PCR using Primer Express 2.0 (Applied Biosystems, CA, USA). The primers fit the requirements related to their length (20–24 bases) and amplicon size (75 and 150 bp). These primer pairs were used to amplify genomic DNA sequences from Jug r genes by conventional PCR. The PCR products were electrophoresed on 2% agarose gels. Amplification of non-walnut DNA extracts from a wide variety of plant samples were also tested to evaluate the specificity of the primers.

2.5. RT-PCR conditions

RT-PCR reactions (20 μL) were performed in a 7900HT Fast real-time PCR System (Applied Biosystems, CA, USA). 10 μL of SYBR Premix Ex Taq™ (Takara, Japan), 2 μmol of primer and variable amount of template DNA were mixed for each reaction and the samples analysed in duplicate. To evaluate performance, two or more non-template controls (NTC) were used in each plate. The PCR program was as follows: initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and primer annealing and elongation at 60 °C for 1 min. The dissociation curves were evaluated to determine primer specificity, and the dissociation studies were carried out on PCR products at a constant temperature increase (60–95 °C) and florescence was recording every 10 s.

The cycle threshold (Ct) value, obtained from 10-fold serial dilutions of walnut genomic DNA, was used to generate standard curves for RT-PCR. The slope of the standard curve (Ct vs. log DNA concentration) indicated the efficiency of each reaction calculated as described in the Applied Biosystem Manual.

2.6. ELISA assay

Detection of walnut protein by immunochemistry was performed with a commercial walnut ELISA kit (AgraQuant walnut assay, Romer Labs, UK) according to the manufacturer's instructions. The microwells were pre-coated with specific antibodies against walnut proteins.

3. Results and discussion

Several real-time PCR protocols have been developed to detect walnut in processed foods using sequences targeting the allergenic protein Jug r 2 (7S globulin) (Brezná et al., 2009; Wang et al., 2009). Nevertheless, as far as we know, there is no RT-PCR method specific for Jug r 1 and Jug r 4, and only one for Jug r 3 (Costa et al., 2013). Therefore, coding sequences for Jug r 1 (2S albumin), Jug r 3 (LTP), and Jug r 4 (11S legumin) were selected as targets to develop a RT-PCR protocol.

By choosing single RT-PCR reactions for each sequence, instead of a multiplex strategy (Pafundo, Gulli, & Marmiroli, 2010), study of the effects of each sequence on the main parameters for RT-PCR (specificity, amplification efficiency, and sensitivity) and a valid comparisons is possible.

3.1. Gene target selection, primer design and sequence analyses

To develop primers for the specific detection of walnut in processed foods, mRNA sequences encoding Jug r 1, Jug r 3, and Jug r 4 allergens were obtained from the NCBI database. These GeneBank sequences were used to design primers pairs to amplify parts of an allergen-coding sequence for the target genes in three cultivars of walnut. DNA from each cultivar was used as a template in conventional PCRs to obtain a specific amplicon that was cloned and sequenced in all cases. The amplicons obtained from Jug r 1 and Jug r 3 were the expected size (277 and 290 bp respectively) because neither contains introns in genomic DNA. However, the Jug r 4 amplicon was longer (1088 bp) than expected because the genomic DNA contains two introns in the target sequence of the primers.

The sequences obtained for each target gene in the different walnut cultivars were aligned with one another and those from GeneBank, showing very high similarity among walnut cultivars (95–100%). These alignments were used to design the specific RT-PCR primers for each of the three target sequences (Table 1). This procedure has been used previously with walnut as well as with other tree nuts to take into account intraspecific polymorphism, which could produce false negative results (Costa, Mafra, Kuchta, & Oliveira, 2012; Costa et al., 2013; Prieto, Iniesto et al., 2014b).

3.2. Optimization of RT-PCR assays

To set up a reliable RT-PCR protocol, the first step is good extraction of DNA with respect to yield and quality values. Both are important requirements to optimise the detection of the target sequences as well as other RT-PCR parameters (Ct, efficiency and R^2 values). Fat and polyphenolic compounds in nuts could act as polymerase inhibitors (Venkatachalam & Sathe, 2006) and should, therefore, be eliminated from the DNA sample. These components absorb at 230 nm while proteins absorb at 280 nm and, therefore, 260/230 and 260/280 ratios were used to test DNA quality.

Table 1
Sequences, melting temperature (T_m), and amplicon size of specific primers used in real-time PCR studies.

Primers	Sequence 5′–3′	T_m (°C)	Amplicon size (bp)
Jug r 1 up	AGGAAATGGAGGAGATGGTGC	59	91
Jug r 1 rp	AACCAGCTTCTGCGAATTTCA	59	
Jug r 3 up	AAGCTGCTGTAATGGGGTCAGG	59.5	99
Jug r 3 rp	ATGGAACCAGCGGTCTTTTTCA	57.2	
Jug r 4 up	GGTCAGCAGGAATATGAGCAGC	60	91
Jug r 4 rp	TGTTGCCTAAGCCCCTCTGTT	60	

The yield, concentration and quality of extracted walnut DNA were optimised by combining CTAB–phenol–chloroform extraction with sample clean up using a silica membrane from the Power Plant DNA Isolation Kit, as previously demonstrated when we evaluated the quantity and the quality of genomic hazelnut DNA extracted with four different methods (Iniesto et al., 2013). This combined method was used for DNA extraction from raw and processed walnuts, and produced a good yield of high quality DNA from both types of samples.

3.2.1. Specificity

The RT-PCR primers generated amplicons from 91 to 99 bp (Table 1). To assess their specificity, DNA from other nuts (peanut, almond, hazelnut, pistachio, and cashew) and plants commonly used as food ingredients (rice, wheat, barley, and rye) were used in a series of RT-PCR experiments. The primer specificity was also confirmed by in silico and using dissociation curves analysis.

The dissociation curves showed a single amplicon when DNA from walnut cultivars were used as a template, and no amplification was observed from other plant samples (Ct values from 33.88 to 37.10, Fig. 1). Similar results have been reported previously for walnuts (Costa et al., 2013) and other nuts (Costa et al., 2012; Pafundo et al., 2010).

Moreover, DNA from other *Juglans* species (*J. nigra* and *J. cinerea*) and other genus in the *Juglandaceae* family (*C. illionensis*) were also used as a template in conventional PCRs to check the specificity of the RT-PCR primers. The amplification products were electrophoresed on agarose gels. All the primers generated positive results with these species tested (Fig. 1 of supplemental material).

Others authors have also described cross-reactivity of walnut PCR primers with *J. nigra* or pecan (*C. illionensis*) (Costa et al., 2014). Broader specificity of the method to *Juglans* spp., other than *J. regia*, could be an advantage because studies *in vitro* (Costa et al., 2014) suggest that some *Juglans* spp. might also cause food allergy in sensitized individuals.

3.2.2. Amplification efficiency and R^2 coefficient

To evaluate RT-PCR performance, three separate DNA extractions of raw walnut were carried out. The DNA was 10-fold serially diluted and 5 μL of each dilution was added to each reaction. The inter-assay reproducibility was investigated by overlapping the three standard curves obtained from different samples in separate experiments. All the standard curve values obtained were optimal in accordance to ENGL acceptance criteria (European Network of GMO Laboratories, 2005): correlation coefficient (R^2) above 0.98 and a PCR efficiency ranging from 90% to 110%, corresponding to a standard curve slope between −3.6 and −3.1. The efficiency values obtained were 99.33% for Jug r 1, 105.08% for Jug r 3, and 102.17% for Jug r 4. The slope varied between −3.21 for Jug r 3 and −3.35 for Jug r 1 (Fig. 2 of supplemental material). Moreover, Ct-values were similar for all the cultivars used (data not shown) and these results suggest the RT-PCR method is highly reproducible.

3.2.3. Sensitivity

The sensitivity of this RT-PCR protocol was assessed with at least three separate DNA extractions of walnut. The limit of detection (LOD) and the limit of quantification (LOQ) were determined using walnut DNA and, as in the efficiency experiments, DNA was 10-fold serially diluted and 5 μL of each dilution added to each RT-PCR reaction. The LOD and LOQ with the primers described here were 2.5 pg (4 genome copies) for Jug r 3, and 25 pg (40 genome copies) for Jug r 1 and Jug r 4. The genome copies were determined using the walnut C-value (0.62 pg) available from the Plant DNA C-values database (Bennett & Leitch, 2012).

According to requirements defined by the European Network of GMO Laboratories (2005), the relative LOD should be determined considering a positive identification of the target at least 95% of the time. To evaluate the sensitivity of this method in food matrices, we performed PCRs using *T. spelta* flour matrices spiked with known amounts of defatted walnut flour (from 0.0005% to 10% w/w). For each concentration, the DNA from at least four separate extractions were analysed by RT-PCR in duplicate. The LOD was determined using the lowest amount of walnut with positive amplification in almost all of the replicates (at least 75%). LOQ was the lowest amplified level within the range of the calibration curve. In our study, the most sensitive primers were for Jug r 3 (Fig. 2), detecting up to 100 mg/kg of walnut (LOD 0.01%, LOQ 0.05%). Costa et al. (2013) achieved a LOD of 50 mg/kg of walnut using conventional RT-PCR, also with Jug r 3 primers. However, the single tube nested RT-PCR developed by the authors increased detection level to 10 mg/kg. In contrast, when DNA was extracted from cakes Costa et al. (2013), the detection level of walnut DNA obtained by conventional RT-PCR was 10 pg, less than the 2.5 pg we achieved with the proposed method. Thus, our approach would appear to be more sensitive with thermally processed samples.

3.3. Effect of high pressure and autoclave treatments

Due to the higher stability of DNA *versus* protein molecules after thermal processing, PCR based methods have been proposed as a powerful alternative tool for allergen detection in processed foods (Diaz-Amigo & Pooping, 2013).

HHP of foods can be used to create new products (texture or taste) or obtain analogue products with minimal effects on flavour, colour, and nutritional value, and without thermal degradation.

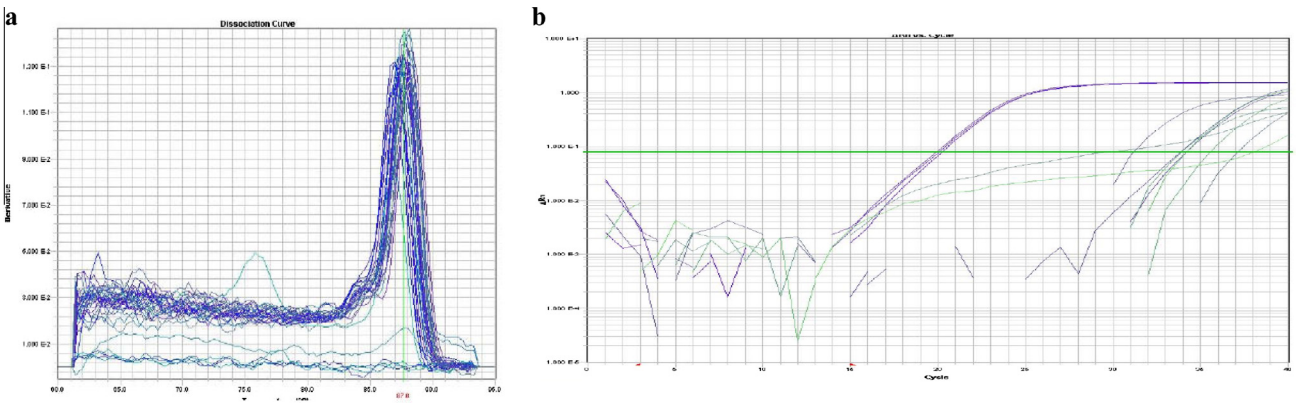

Fig. 1. RT-PCR amplification with SYBR Green dye target Jug r 3 gene of walnut. (a) Melting curve. (b) Ct values, the walnut cultivars showed Ct values around 20 and the other species hazelnut, almond, peanut, wheat, barley, rye ranged from 33.88 to 37.10.

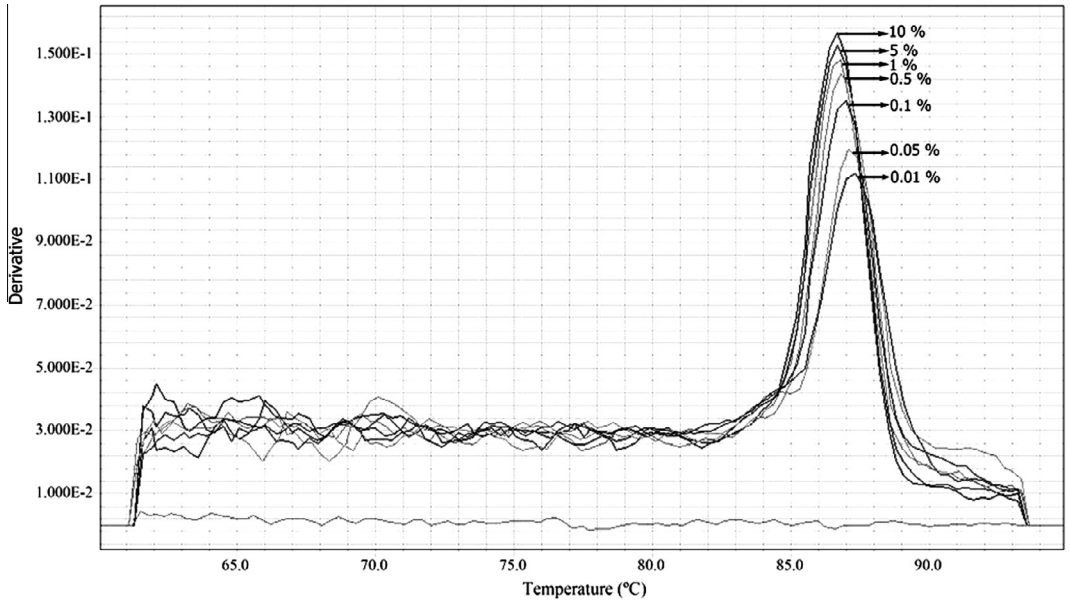

Fig. 2. Melting curve analysis obtained by Real time PCR with SYBR dye from mixtures containing 10%, 5%, 1%, 0.5%, 0.1%, 0.05% and 0.01% of walnut flour. The target gene was Jug r 3.

The effect of HHP on allergenicity has been investigated mainly by analysis of changes in protein structure (Somkuti & Smeller, 2013). HHP can unfold proteins at around 500 MPa, but the pressure needed can vary from protein to protein in the range 100 MPa to 1 GPa or higher in special cases (Somkuti & Smeller, 2013). More specifically, the effects of HHP on food allergenicity have been studied in beef, apple, celery (Husband et al., 2011; Johnson et al., 2010) and in nuts, such as peanut, walnut and hazelnut (Cabanillas et al., 2014; Johnson et al., 2010; Prieto, Burbano et al., 2014a).

In order to study the effect of HHP and thermal treatments on the detection capacity of the proposed RT-PCR, a calibration curve was performed for each primer and treatment. Three DNA extracts were obtained from each treatment and analysed in duplicate. A linear regression was obtained in all cases by plotting Ct values against the logarithm of walnut DNA concentration (Table 2).

HHP did not affect the detection of walnut DNA, and we were able to identify up to four genomes from walnut following all the HHP treatments using Jug r 3 primers. Moreover, as Table 2 shows, the slope, efficiency, and correlation coefficient values of all calibration curves indicated a high performance. The effect of HHP on the detection of allergenic coding sequences from other tree nuts was reported recently by our group (Iniesto et al., 2013). Our findings with hazelnuts demonstrated that HHP did not affect the DNA extraction yield, quality, integrity and amplification. However, in almond (*Prunus dulcis*), these treatments affected the detection of DNA; amounts of almonds detected in these samples was higher than in untreated samples due to DNA fragmentation (Prieto, Iniesto et al., 2014b). Similar differences have been reported elsewhere for allergenic proteins following HHP treatments. Several authors concluded that stability, in response to pressure, varies depending on the protein secondary structure (Johnson et al., 2010; Somkuti & Smeller, 2013). Also, this effect seems to depend on the specific material studied, for example hazelnut *versus* almond. However, only a few reports have analysed the influence of HHP treatments on RT-PCR performance (Iniesto et al., 2013; Prieto, Iniesto et al., 2014b) and, therefore, more studies at this basic level are needed.

Thermal treatment of walnut flour dramatically reduced the ability to detect walnut DNA (Table 2). This reduction in sensitivity increased with temperature (from 121 °C to 138 °C) and time (from 15 min to 30 min). RT-PCR sensitivity was also decreased in autoclaved samples. After 15 min at 121 °C, the reduction was 10× compared to control, and 100× when the time was increased to 30 min (Fig. 3 of supplemental material). At 138 °C, detection was difficult and the quantification was not possible. Similar results have been reported for peanut (Scaravelli et al., 2009), hazelnut (Iniesto et al., 2013) and almond (Prieto, Iniesto et al., 2014b). Moreover, some thermal processing methods induce fragmentation and/or degradation of genomic DNA causing a marked drop in DNA detection of GMOs using PCR (Ballari & Martin, 2013; Gryson, 2010).

To determine if the autoclave treatment affected the amounts and/or integrity of walnut DNA, both parameters were evaluated. DNA integrity, analysed using agarose gel electrophoresis, produced results compatible with the degradation of DNA after heating under pressure. Our data demonstrated that thermal treatment under pressure affected walnut DNA amplification, as previously described in hazelnut and almond (Iniesto et al., 2013; Prieto, Iniesto et al., 2014b). However, Scaravelli et al. (2009) concluded that the extraction of peanut DNA decreased considerably as thermal treatment (backing) increased, but DNA integrity and quality were not affected. Nevertheless, when Costa et al. (2013) analysed food samples containing walnuts, baked at 200 °C for 20 min, there was no effect on detection of walnut DNA. LOD and LOQ values were similar to those for unprocessed samples. Our results suggest a stronger effect of thermal treatment on DNA integrity when heat and pressure are applied. Under increased pressure, DNA integrity is affected strongly and the DNA fragment size was shorter than 100 bp. Such fragments are not long enough to amplify, as has been reported in GMO detection (Ballari & Martin, 2013; Gryson, 2010).

3.4. Applicability on commercial food samples

The applicability of the developed RT-PCR method for determining walnut allergens in foods was investigated by analysing 12 commercial foodstuffs, comprised of snacks, chocolates, cookies, nougat, etc. Two of these products were labelled as "contains walnuts", seven indicated that the product "might contain traces of nuts" and three did not declare any walnut content. All the com-

Table 2
Real-time PCR results for the detection of Jug r 3 in walnut defatted flour after high hydrostatic pressure HHP (a) and autoclave treatments (b). The rows shows the mean cycle threshold (Ct) ± standard error.

(a) ng Walnut DNA/genome copies	HHP treatment				
	Control	HHP300 MPa	HHP400 MPa	HHP500 MPa	HHP600 MPa
25 ng/40323	21.79 ± 0.18	20.88 ± 0.03	21.23 ± 0.01	21.91 ± 0.02	21.37 ± 0.14
2.5 ng/4032	25.00 ± 0.19	24.43 ± 0.03	24.34 ± 0.02	24.99 ± 0.03	24.36 ± 0.05
0.25 ng/403	28.26 ± 0.20	27.54 ± 0.03	27.70 ± 0.1	28.27 ± 0.05	27.69 ± 0.05
0.025 ng/40	31.39 ± 0.46	30.37 ± 0.03	30.63 ± 0.09	31.34 ± 0.14	30.92 ± 0.05
0.0025 ng/4	32.65 ± 0.72*	32.10 ± 0.10*	32.93 ± 0.68*	nd	33.45 ± 0.09*
R^2	0.999	0.997	0.999	0.999	0.999
Slope	−3.21	−3.16	−3.16	−3.16	−3.20
Efficiency (%)	105.08	107.33	107.31	107.38	105.34
(b) ng Walnut DNA/genome copies	Autoclave treatment				
	Control	121 °C 15 min	121 °C 30 min	138 °C 15 min	138 °C 30 min
25 ng/40323	21.79 ± 0.18	25.12 ± 0.09	28.97 ± 0.04	32.79 ± 0.13*	32.84 ± 0.14*
2.5 ng/4032	25.00 ± 0.19	28.43 ± 0.02	31.61 ± 0.02	33.02 ± 0.47*	33.48 ± 0.57*
0.25 ng/403	28.26 ± 0.20	31.39 ± 0.12	33.13 ± 0.82	33.92 ± 0.29*	33.81 ± 0.42*
0.025 ng/40	31.39 ± 0.46	34.90 ± 0.9*	33.82 ± 0.27*	32.85 ± 0.47*	–
0.0025 ng/4	32.65 ± 0.72*	–	–	–	–
R^2	0.999	0.999	0.93	0.07	0.1
Slope	−3.21	−3.23	−2.08		
Efficiency (%)	105.08	103.91	202.13		

Footnotes: no detected (−), no determined (nd), *detection is possible but the Ct value are not in the calibration curve.

mercial food samples were analysed by ELISA, and the results compared in Table 3.

The study used all three primer pairs, Jug r 1, Jug r 3 and Jug r 4, and detected the presence of walnut according to the declaration on the label, even at trace levels. The utilisation of this real-time protocol, based on three different target sequences, reduces the probability of false negatives and positives. Jug r 3 probes were the most sensitive for detecting walnut traces in the commercial food samples selected.

The correspondence between ELISA results, and those obtained with RT-PCR using Jug r genes, was 10 out of 12 food products analysed, confirming the reliability of this RT-PCR method. In two samples, cereal snack II and chocolate with cereal, the signal obtained with the ELISA was below the LOQ (2–60 ppm). However, traces of walnut DNA were detected in both samples using Jug r 3, and Jug r 4 in one. The detection of walnut DNA in a non-walnut-declared sample, such as cereal snack II, the results of which by ELISA were negative, confirmed the higher sensitivity of the RT-PCR method developed. Thus, the procedure described here could be more reliable than the ELISA tested.

In this work, we proposed a new molecular method targeting a gene encoding the allergenic protein Jug r 3 of walnut. Jug r 3 belongs to the lipid transfer proteins (LTP), and is classified as a major allergen related to severe clinical presentation (systemic reactions) in walnut allergic individuals (Pastorello et al., 2004). The suggested approach is based on RT-PCR following successful application with other tree nuts, such as hazelnut (Costa et al., 2012; Iniesto et al., 2013) and almond (Costa et al., 2013; Prieto, Iniesto et al., 2014b).The results indicated that the method developed is specific, reliable, and sensitive, with a low LOD (2.5 pg of walnut DNA).

The main interest and novelty are the findings from processed walnuts samples, because of the scarcity of studies on how HHP and autoclave treatments can affect the DNA quality required for PCR amplification. Thermal treatment combined with pressure (autoclaving) reduced yield (integrity and quality) and amplification of walnut DNA. HHP without heating did not degrade the DNA and, therefore, had no effect on detection. This RT-PCR is accurate, sensitive, and specific for the detection of walnut in processed commercial foods. The complexity of the processing methodologies in the food industry makes understanding of their impact at the molecular level a priority. Ultimately, the goal is to develop techniques capable of detecting traces amounts of allergens in food and move towards knowledge-based management of allergen risk as well as assuring consumer safety.

Table 3
Walnut detection in commercial food products analyzed through real-time PCR (detection was indicated as Ct value ± standard error and no detected as −) and ELISA (+ = detected; − = non-detected).

Commercial foods	Walnut declaration	Jug r 1	Jug r 3	Jug r 4	ELISA
Salami with walnuts	Contain	nd*	27.28 ± 0.38	nd*	+
Pastillo nuts	Contain	nd*	23.81 ± 0.18	nd*	+
Chocolate with hazelnut	Might contain nuts	30.64 ± 0.31	28.97 ± 0.50	25.67 ± 0.63	+
Cereal snack I	Might contain nuts	nd*	30.24 ± 0.17	nd*	+
Cereal snack II	Might contain nuts	–	30.69 ± 0.45	30.50 ± 1.04	−
Chocolate	Might contain nuts	–	–	–	−
Chocolate with cereal	Might contain nuts	–	31.37 ± 0.15	–	−
Nougat	Might contain nuts	28.62 ± 0.47	29.02 ± 0.17	24.60 ± 0.45	+
Cookies with cereal and chocolate	Might contain nuts	nd*	30.62 ± 1.28	nd*	+
Cereal snack III	Not declared	29.04 ± 0.23	27.93 ± 1.07	24.03 ± 0.73	+
Chocolate snack with cookie	Not declared	29.56 ± 0.95	27.16 ± 1.40	25.74 ± 0.87	+
Chocolate cookie	Not declared	30.36 ± 0.39	26.74 ± 1.37	26.16 ± 0.80	+

* nd = non-determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.01.132.

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Appendix 4. Supplementary Material

Supplementary information

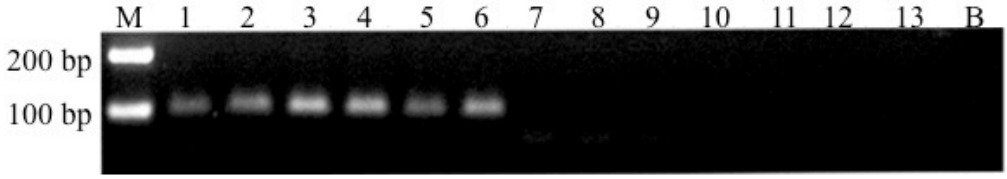
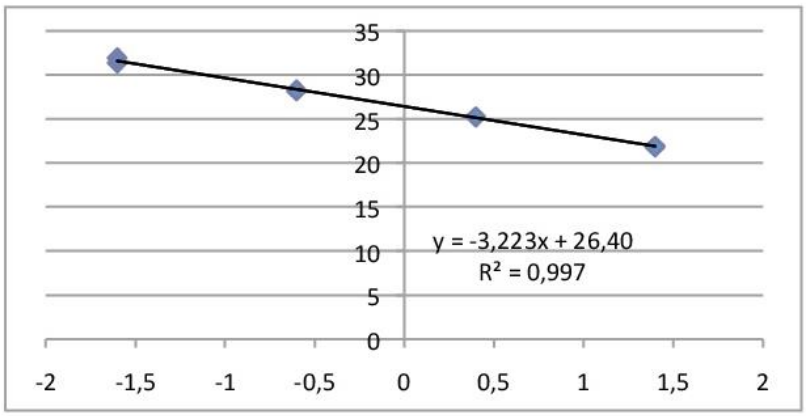
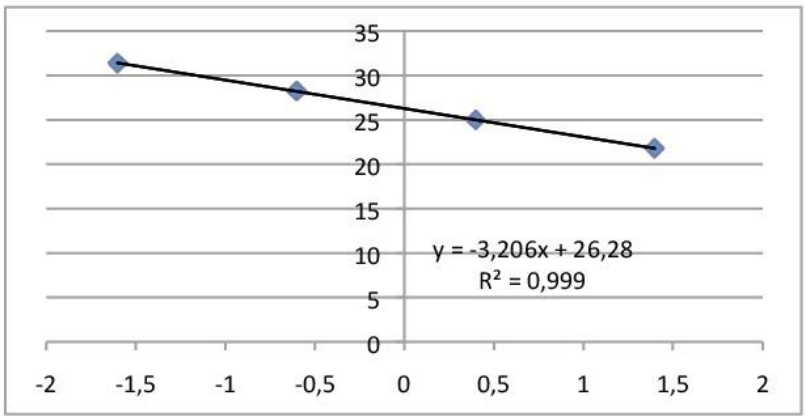


Figure 1. Specificity of Real-time PCR using Jug r 3 primers assayed in different species. Lanes 1−3: *Juglans regia* cv Chandler, California and Oprean; 4: *Carya illinoensis*; 5: *Juglans cinerea;* 6: *Juglans nigra x cinerea*; 7: *Corylus avellana*; 8. *Prunus dulcis*; 9: *Pistacia vera*; 10: *Anacardium occidentale*; 11: *Triticum spelta*; 12: *Oryza sativa;* 13: *Arachis hypogaea* B: negative control. Electrophoresis in 2% (w/v) agarose gel. M: 100 pb ladder.

a



b



c

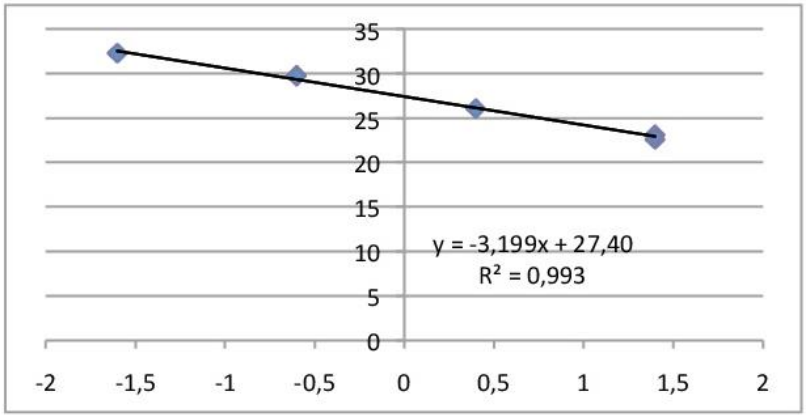


Figure 2. Calibration curve of Jug r primer pairs a: Jug r 1, b: Jug r 3, and c: Jug r 4. The Ct value is represented (y axis) against walnut content log (x axis). The linear regression equation and the correlation coefficient (R^2) are given.

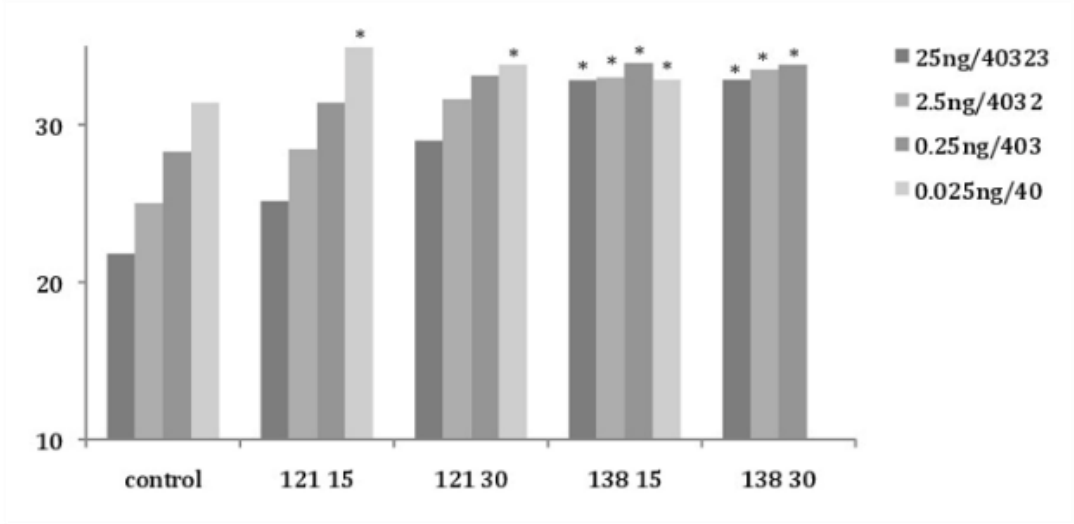


Figure 3. Comparation of Ct values obtained with Jug r 3 RT-PCR of 10-fold serially diluted walnut DNA (25ng to 25pg) from different samples 1: Control raw samples; 2: Autoclaved (120 kPa, 15´); 3: Autoclaved (120 kPa, 30´); 4: Autoclaved (260 kPa, 15´); 5: Autoclaved (260 kPa,30´)

* Detection is possible but the Ct value are not in the calibration curve.

Artículo 5

DETECCIÓN DE SECUENCIAS CODIFICANTES DE ALÉRGENOS DE PISTACHO EN ALIMENTOS: UNA COMPARACIÓN DE DOS ESTRATEGIAS DE PCR EN TIEMPO REAL.

DETECTION OF PISTACHIO ALLERGEN CODING SEQUENCES IN FOOD PRODUCTS: A COMPARISON OF TWO REAL TIME PCR APPROACHES.

El etiquetado de pistacho en alimentos es obligatorio por lo que es recomendable el desarrollo de metodologías analíticas adecuadas para detectar este fruto seco en alimentos procesados. En este trabajo, se prueban y comparan dos químicas (SYBR®Green y sondas LNA) para detectar pistacho mediante PCR en tiempo real. Se han amplificado y clonado las secuencias codificantes de los alérgenos Pis v en diferentes variedades de pistacho, y se han diseñado cebadores y sondas específicos para cada alérgeno. De acuerdo con los resultados, la PCR en tiempo real basada en sondas LNA parece ser la más sensible y específica, alcanzando 10 mg/kg de pistacho. Este método se empleó para evaluar el efecto de la temperatura y/o la presión en la detección de ADN de pistacho. Los datos muestran una reducción en la amplificación de pistacho después del tratamiento térmico con presión, sin embargo, no se observa este efecto tras aplicar cocción. Se han analizado 14 alimentos comerciales, comparando el resultado con un kit ELISA, para estudiar la aplicabilidad del método desarrollado.

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Detection of pistachio allergen coding sequences in food products: A comparison of two real time PCR approaches

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ABSTRACT

The labelled of pistachio on food products is mandatory and, as a consequence, the development of suitable analytical methodologies to detect this nut in processed foods is advisable. In this work, two different qPCR assays to detect pistachio, SYBR®Green and locked nucleic acid (LNA) probes, are tested and compared. Pis v allergen coding sequences have been amplified and cloned in different pistachio varieties, and specific primers and probes for each allergen have been designed. According to our results, LNA probe-real time PCR appears to be the most sensitive and specific method, reaching 10 mg/kg of pistachio. The effect of temperature and/or pressure on pistachio DNA detection was also analysed by LNA probe-based qPCR. Data showed a reduced amplificability of pistachio after thermal treatment under pressure, nevertheless, this effect was not observed after boiling. The applicability of this method has been studied by analysing 14 food products and by comparison with a commercial ELISA kit.

1. Introduction

Food allergy is considered a relevant health problem all over the world, and its prevalence is difficult to establish because it varies by country and by food, being estimated to affect close to 5% of adults and 8% of children (Sicherer & Sampson, 2014). The most common foods associated with allergenic reactions are fish, peanuts, soybeans, milk, eggs, crustacean, wheat and tree nuts. The importance of tree nuts allergy is not only for the severity of reactions but also for the prevalence in population (Burney et al., 2014; McWilliam et al., 2015). Consumption of Anacardiaceae nuts, as pistachio and cashew, is rising over the last years especially as snack foods or as ingredients in several food dishes. California, followed by other countries such as Iran and Turkey are producers of pistachio (Ahmad, Ferguson, & Southwick, 2003). In Europe, Greece, Italy and Spain are increasing the export of this nut (USDA, EU-28 Tree Nuts Annual, 2013). Organoleptic properties of this edible tree nut are really valued and many nutritional benefits of pistachio have been reported, as a high content of essential fatty acids, proteins and other healthy components (Dreher, 2012). Pistachio nuts intake has a favourable cardiovascular impact according to the Dietary Guidelines for Americans. Pistachio is included on the list of allergens in Europe (Regulation EU No 1169/2011/EC, OJEU, 2011), and it must be indicated on food labels. Because of the high price of pistachio nuts, its presence in processed products is normally due to adventitious contamination in the food facility, not to a fraudulent substitution, and it represents an important health risk for sensitized patients.

Five pistachio proteins are identified as allergens in the allergen WHO-IUIS list (WHO/IUIS Allergen Nomenclature Database): Pis v 1, Pis v 2, Pis v 3, Pis v 4 and Pis v 5. Pis v 4 is a manganese superoxide dismutase (Noorbakhsh et al., 2010a) and the rest of them represent the major seed storage protein constituents of the nuts (Roux, Teuber, & Sathe, 2003). They belong to three protein families, 2S albumins (Pis v 1), vicilins (7S globulins as Pis v 3), and legumins (11S-13S globulins as Pis v 2 and Pis v 5), representing globulins and albumins the 66% and 25% of the total protein, respectively (Ahn, Bardina, Grishina, Beyer, & Sampson, 2009;

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Shokraii & Esen, 1988; Willison et al., 2008).

The necessity to guarantee the legislation compliance and ensure the consumer protection has triggered the development of reliable and sensitive analytical techniques in order to detect food allergens, even traces, in processed products. The most common method to determine the presence of specific food allergens is enzyme-linked immunosorbent assay (ELISA), and there are many ELISA tests for pistachio on the market. However, the structure of proteins as well as their IgE-binding capacity can be affected by food processing, and consequently, techniques based on protein detection could be altered (Cabanillas et al., 2012, 2014; Sathe et al., 2009). DNA-based methodologies for detection of allergenic food traces have been increasingly proposed as a specific and sensitive alternative to protein-based ones. In the last years, several studies have been carried out to develop a reliable and sensitive method of real time PCR (or qPCR) for detecting food allergen sequences using DNA targets, which normally present higher thermal stability than proteins (Costa, Ansari, Mafra, Oliveira, & Baumgartner, 2014).

Many works describing a real time PCR method to detect food allergens have been reported using SYBR-Green as intercalating dye (Linacero et al., 2016; Pafundo, Gull, & Marmiroli, 2010; Prieto, Iniesto et al., 2014a). The reduced specificity of this detection chemistry, which binds double strand DNA, can be considered as a drawback, and hydrolysis probes-based PCR is a suitable alternative for detecting specific sequences, since finally three DNA sequences are incorporated to the PCR reaction. Locked nucleic acids (LNA) are DNA nucleotide analogues with higher affinity and specificity to bind complementary nucleic acids, thus nonspecific amplification can be greatly avoided (Latorra, Arar, & Hurley, 2003). This method has been proposed as a good alternative to others, even as classic TaqMan probes (Gašparič et al., 2010; Salvi, D'Orso, & Morelli, 2008). In this work, two real time PCR approaches, based on SYBR-Green chemistry and the use of locked nucleic acid (LNA) probes, are compared to obtain the most appropriated to detect pistachio allergen coding sequences.

Pistachios are usually thermally processed before consumption, and real time PCR methods should be able to reach optimal sensitivity values for detecting a specific pistachio target in processed food samples. Only a few works have been published for the specific detection of pistachio DNA in processed foodstuff by different PCR approaches and selected targets (Barbieri & Frigeri, 2006; Brežná, Dudášová, & Kuchta, 2008; Köppel, van Velsen-Zimmerli, & Bucher, 2012; Lopez-Calleja, de la Cruz, González, García, & Martín, 2014). To our knowledge, any work has been performed for the detection of pistachio allergen coding sequences, and nuclear DNA targets are appropriate candidates for developing a quantitative real time PCR assay (Prado, Boix, & Holst, 2012). This is important in order to analyse the consequences that food processing/treatments might exert on DNA molecules and its amplification by real time PCR.

Several works about the processing influence on DNA detection and quantification by real time PCR have been published in the last years. A negative effect of severe heat treatments on DNA detection and even fragmentation and/or degradation of DNA molecules has been reported (Costa, Oliveira, & Mafra, 2013; Gryson, 2010; Hildebrandt & Garber, 2010; López-Andreo, Aldeguer, Guillén, Gabaldón, & Puyet, 2012; Scaravelli, Brohée, Marchelli, & Van Hengel, 2009). Our group has analysed the effect of high hydrostatic pressure (HHP) and autoclave in the detection of tree nuts such as hazelnut, almond and walnut by real time PCR methodology (Iniesto et al., 2013; Linacero et al., 2016; Prieto, Iniesto, et al., 2014a). The findings showed that the high pressure affects differently depending on the tree nut sample and, nevertheless, autoclave processing influences DNA integrity and amplification in all these nuts.

The present study is aimed to set up a reliable and suitable real time-based detection assay for pistachio allergen coding sequences. In addition, the goal is to analyse the influence of high temperature and/or high-pressure treatments on Pis v 1 detectability by LNA probe-based real time PCR.

2. Materials and methods

2.1. Samples

Five varieties of *Pistacia vera* L. nuts (Kerman, Sirora, Aegina, Mateur and Larnaka) were obtained from the collection of Institut de Recerca i Tecnologia Agroalimentaries (IRTA-Mas de Bover, Tarragona, Spain). Specificity studies were carried out with other plant species commonly used as food ingredients (cashew, mango, orange, lemon, peanut, walnut, wheat, rye and peach).

For the preparation of spiked samples mixtures containing 100,000, 10,000, 1000, 100, 10, 5, 1, 0.5 mg/kg (from 10% to 0.00005% w/w) of defatted raw pistachio flour *P. vera* var. Kerman in spelt wheat (*Triticum spelta* L.) were prepared in a final weight of 100 g. The first mixture containing 10% of pistachio was elaborated by adding 10 g of defatted pistachio flour to 90 g of wheat flour, followed by 10-fold dilutions up to 0.5 mg/kg. All samples were completely homogenized using a kitchen robot (Thermomix 31-1, Vorwerk Elektrowerke, GmbH & Co. KG, Wüppertal, Germany) at maximum speed. Food samples (chocolate, cereal bar, biscuit, meat and sauce) used in this work were purchased from different local stores, ground using the kitchen robot and stored at −20 °C until their use.

2.2. Boiling and autoclave treatments

One hundred grams of pistachio nuts var. Kerman were immersed in distilled water (1:5 w/v) and then boiled for 30 and 60 min or autoclaved using a Compact 40 Benchtop autoclave (Priorclave, London, UK) at 121 °C (120 kPa) and at 138 °C (256 kPa) for 15 and 30 min. Raw and treated pistachios were freeze-dried (Telstar Cryodos freeze-drier), defatted with *n*-hexane (34 ml/g of flour) and milled using a Cyclotec 1093 Sample Mill, with a sieve of 1 mm.

As described before, binary mixtures containing from 10% to 0.001% (w/w) (100,000, 10,000, 1000, 100, 10 mg/kg) of defatted treated pistachio flour in spelt wheat were also performed. All samples were stored at 4 °C.

2.3. DNA extraction

One hundred milligrams of each spiked sample were homogenized with 1000 μl of Cetyl trimethylammonium bromide (CTAB) with 1% of polyvinylpyrrolidone (PVP) and 3 μl of 25 mg/ml RNAse, and were incubated at 65 °C for 30 min, shaking at intervals of 10 min. Then, 800 μl of chloroform were added to the sample before centrifugation at 15,871g for 10 min. Genomic DNA was obtained from 800 μL of aqueous supernatant following the Power Plant DNA isolation kit (MoBio, CA USA) and finally eluted in 100 μL of 10 mM Tris pH 8. DNA from food samples (200 mg) was obtained using NucleoSpin kit (Macherey-Nagel, Düren, Germany) following the manufacturer instructions with minor modifications for chocolate-based products. In order to improve the quantity and quality of the extracted DNA, the incubation time at 65 °C with stirring was extended up to 1 h and then 25 mg/ml RNAse was added and incubated for 10 min at 37 °C. The number of washing steps with ethanol and guanidine hydrochloride was also increased, following the recommendations from Costa and collaborators (Costa, Melo, Santos, Oliveira, & Mafra, 2015). Quality of isolated DNA from all

the samples was evaluated on 0.8% TBE agarose gels. DNA quality and concentration were determined spectrophotometrically with NanoDrop ND-1000 spectrophotometer (Thermo-Fisher, Waltham, MA, USA), taking into account the values obtained by measuring absorbance at 230, 260 and 280 nm.

2.4. Conventional PCR, cloning, sequencing and real time PCR primer design

All primers have been produced by Integrated DNA Technologies (IDT, Coralville, Iowa, USA). Primers for conventional PCRs were designed with Oligo Primer Analysis Software, using the coding sequences of different allergens as a target (Table 1 Supplementary material): Pis v 1 Pis v2, Pis v 3, Pis v 4 and Pis v 5 genes. The requirements established for primers were length (20–25 bases) and amplicon size (95–115 bp). The conventional PCR reactions were performed with DNA from the pistachio varieties Kerman, Sirora, Aegina, Mateur and Larnaka. These reactions were carried out in 20 μl, containing 25 ng of DNA, 0.25 μM of each primer and 1X FastStar PCR Master Mix (Biotools, Loganholme, Australia). SensoQuest LabCycler (Progen Scientific Ltd, Germany) was programmed with an initial denaturation step at 95 °C, 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, elongation at 72 °C for 1 min and last step at 72 °C for 6 min. The resulted amplicons were cloned into the pCR™4-TOPO® Vector using TOPO® TA Cloning® Kit (Invitrogen, Inc., UK) following the manufacturer's instructions. Plasmid DNA was purified using Plasmid DNA purification kit (Biotools, Loganholme, Australia) and sequenced in an ABI PRISM 3700 sequencer (Applied Biosystems, Foster City, CA, USA) from the Genomics Service (Universidad Complutense de Madrid, Spain).

Software Bioedit (Ibis Biosciences, Carlsbad, CA, USA) was employed to edit DNA sequences. The edited sequences were compared with those present in the GenBank (NCBI) nonredundant nucleotide (nr/nt) databases, through BLAST. ClustalW algorithm (Thomson, Desmond, & Gibson, 1994) was used to carry out the alignments between sequences of the allergen gene from the five pistachio varieties and homologue sequences from another plant species, available in the GenBank. The conserved sequences between pistachio varieties were used to design specific primer pairs for each target sequence (Pis v 1, Pis v 2, Pis v 3, Pis v 4 and Pis v 5) by Primer Express 2.0 (Applied Biosystems, CA, USA) for SYBR-Green chemistry-based real time PCR and by Universal Probe Library for Human (Roche, Germany) for probe-based qPCR. The primers fit the requirements related to their length (20–25 bases) and amplicon size.

2.5. Real-time PCR

Real time PCR reactions were performed with 7900HT Fast Real-Time PCR (Applied Biosystems, CA, USA). SYBR-Green chemistry based qPCR was carried out in a final reaction volume of 20 μl, containing 10 μl of SYBR® Premix Ex Taq (Takara, Japan), 0.25 μM of each primer and 5 μl of template DNA at different concentrations. The employed PCR program was: an initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and primer annealing and elongation at 62 °C for 1 min. Locked nucleic acid probe-based real time PCR was performed in a volume of 20 μl with 10 μl of TaqMan®Gene Expression Master Mix (Applied Biosystem, CA, USA), 0.25 μM of each primer, 0.1 μM of LNA probe and 5 μl of extracted DNA at different concentrations. Different final concentrations of primers (from 250 nM to 900 nM) and probe (from 50 nM to 250 nM) were tested to obtain the proper performance. This real time PCR was run under standard conditions, which include an initial denaturation at 95 °C, for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and primer annealing and elongation at 60 °C for 1 min. At least two experimental and three technical replicates were analysed, and two non-template controls (NTC) were included in each plate to check the PCR performance. DNA sequences used for SYBR-Green and probe-based real time PCR are listed in Tables 1 and 2

The cycle threshold (Ct) values obtained from spiked samples of raw pistachio genomic DNA was used to generate the standard curves for real-time PCR. The efficiency ($10^{(-1/slope)}$-1) of each reaction was calculated from the slope of the standard curve (Ct vs log DNA concentration) as indicated in the Applied Biosystems manual. This value, as well as specificity and limit of detection (LOD) from both methods, were compared. The specificity of all primers was assessed by testing the amplification of non-pistachio DNA extracts from a wide variety of plant samples. For SYBR-Green chemistry, a curve with raw pistachio DNA was also performed for all Pis v primers.

The effect of thermal processing over PCR amplification, whether applying pressure and just boiling, was analysed using spiked samples of processed pistachio flour in wheat (from 100,000 to 10 mg/kg) by LNA-based method. Applicability of this assay was tested by the evaluation of 14 commercial foodstuffs. In order to avoid false negative results, all extracted DNA was evaluated by probe-based real time PCR or conventional PCR using 18S primers (Table 2), designed with the information available in GenBank database (accession number GQ380689.1), as positive amplification control.

2.6. ELISA

Pistachio protein detection by immunochemistry was performed using a commercial pistachio ELISA kit a quantification range from 1 mg/kg to 40 mg/kg (AgraQuant pistachio assay, Romer Labs, UK) according to the manufacturer's instructions. The kit includes microtiter strips pre-coated with specific polyclonal antibody against pistachio proteins.

2.7. Statistical analysis

The significance of differences between the Ct values of each spiked level within the same treatment (untreated and treated by boiling and autoclave processing) was evaluated with analysis of variance (ANOVA) using Statgraphics Centurion Programme. Significant differences were considered when $p < 0.05$. Previously, all data were assessed for normality by Kolmogórov-Smirnov test.

3. Results and discussion

In this work pistachio allergens-coding sequences, Pis v 1 (2S albumin), Pis v 2 (11S globulin), Pis v 3 (7S vicilin), Pis v 4 (SOD) and Pis v 5 (11S globulin), have been used as targets to develop a reliable

Table 1
DNA sequences of the primer pairs used for Sybr-Green real time PCR.

Primers	Length	Sequence (5'→3')	Fragment length (bp)
Pis v 1 fw	23	TCAAGCACTGCCAAATGTACGTG	96
Pis v 1 rev	22	TGAAGCATTGTTGTCGCTGGTT	
Pis v 2 fw	17	CGGAATTGCCAACGTCT	103
Pis v 2 rev	18	GGAGGGTGATGTCATTGC	
Pis v 3 fw	20	AGAGGAATGGGGAAGCGGTC	109
Pis v 3 rev	20	CCCTCCGTCTTGTCTCTCGC	
Pis v 5 fw	21	GCTGGTAACCCAGAAGATGAG	77
Pis v 5 rev	23	AGATTTCTGTTGGCTTTGTCTAC	

Table 2
DNA sequences of the primers and probes (from Roche Universal Probe Library, Human Catalogue) used for probe-based real time PCR.

Primers	Length	Sequence (5′→3′)	Fragment length (bp)
Pis v 1 forward	21	GGCAAAGCTCGTACTTCTCCT	81
Pis v 1 reverse	20	TCCACAGTAGCGCGGTAGAT	
Pis v 1 probe	8	5′ FAM-AGGCAGAG-Q 3′	
Pis v 2 forward	21	GCTAGCCAGAAGACTGCAGAA	65
Pis v 2 reverse	21	GGTCCTCCTGAACTCTGACAA	
Pis v 2 probe	8	5′ FAM-CTGCCTTC-Q 3′	
Pis v 3 forward	19	CGCTTCAGGTGTCAGGAGA	92
Pis v 3 reverse	18	TGTTCCTCATCGCCTTCC	
Pis v 3 probe	9	5′ FAM-AGAAGAGCA-Q 3′	
Pis v 5 forward	18	ATTTTCGCGCTTCGACAC	77
Pis v 5 reverse	23	TTTTGGAGCTGCTTTACAAGACT	
Pis v 5 probe	8	5′ FAM-GGCTGAGG-Q 3′	
18S forward	20	CGCGAGAAGTCCACTAAACC	64
18S reverse	21	CCTACGGAAACCTTGTTACGA	
18S probe	8	5′ FAM-GGAAGGAG-Q 3′	

real time PCR protocol to detect pistachio traces in food. Previously others authors (Brežná et al., 2008; Ehlert, Demmel, Hupfer, Busch, & Engel, 2009; Köppel et al., 2012; Lopez-Calleja et al., 2014) have used the real time PCR approach to detect this nut, but the target sequences have been others than the coding sequences indicated above.

In order to develop primers for the specific detection of pistachio, mRNA sequences from these allergen-coding genes were obtained from the NCBI database and were used to design primer pairs to amplify partial allergen-coding sequences in five varieties of pistachio (Kerman, Sirora, Aegina, Larnaka and Mateur), reducing false negatives due to inter-variety polymorphisms (D'Andrea et al., 2011). Subsequently, DNA from each variety was used as a template in conventional PCRs to achieve a specific amplicon that was cloned and sequenced, obtaining the expected amplicon size for Pis v 1, which not contains introns in genomic DNA. Pis v 2, Pis v 3, Pis v 4 and Pis v 5 amplicons were longer than expected because of the presence of introns (Table 1 supplementary material). The sequences obtained for each Pis v gene in all the varieties and the homologue sequences from related species which showed identity value higher to 66% at Genbank, were aligned. These alignments were used to design specific primer pairs for SYBR-Green and probe-based real time PCR. Allergen-coding sequences are a good candidate because are present in a constant and low copy number in the species genome, thus the use of these kind of genes is recommended for quantitative assays (Prado, Boix, & Holst, 2012). Highly repetitive sequences and variable copy number as ribosomal, mitochondrial and chloroplast DNA could add unreliability of the methodology although also improve the assay sensitivity (Brezna & Piknova, 2013, chap. 18).

3.1. Efficiency, sensitivity and specificity of Pis v primers in SYBR-Green PCR

In a real time PCR assay, the slope of the standard curve is used to calculate the efficiency of the amplification reaction. A slope of −3.32 corresponds to a 100% efficient PCR reaction, and R^2 describes the correlation between the amount of pistachio and the cycle threshold (Ct) in the standard curve. Efficiency of Pis v primer pairs, listed in Table 1, using SYBR-Green chemistry was tested using two different curves, one of them based on a 10-fold serially diluted pistachio DNA curve and the other one on a food matrix, using *T. spelta* flour spiked with known amounts of defatted pistachio flour, from 100,000 to 0.5 mg/kg. In all the cases, at least two different DNA isolations and two technical replicates were evaluated. Efficiency values within the acceptable criteria (90%–110%) were obtained for both analysed curves with Pis v 1 (92.55% for DNA curve and 108.15% for spiked curve) and Pis v 2 (108.90% for DNA curve and 103.03% for spiked curve) (Fig. 1A and B supplementary material). The sensitivity for these two primer pairs was good, establishing the limit of detection (LOD) in 12.5 pg of pistachio DNA and 100 mg/kg or 10 mg/kg in mixtures of raw pistachio flour in wheat, for Pis v 1 and Pis v 2 primers respectively. However, we found some technical problems with SYBR-Green using Pis v 1 primers: the dissociation curves from *Triticum spelta* wheat overlapped with the pistachio one (Fig. 1A), and heterogeneous products were observed by agarose gel analysis. Regarding Pis v 2 primers, other Anacardiaceae species, as cashew, was not distinguished from pistachio DNA (Fig. 1B) even when sequences from these related species were taken into account in the primer design process. Real time PCR performance with Pis v 3 and Pis v 5 primers was not suitable regarding sensitivity (limit of detection and quantification) and specificity, since DNA from species as cashew, mango and other nuts was also detected (data not shown). That fact could be an evidence of the lack of specificity for this SYBR-Green assay.

Finally, probe-based real time PCR was tested for Pis v primers, indexed on Table 2, and results for Pis v 1 primer set are explained in this work.

3.2. Efficiency, sensitivity and specificity of Pis v primers in LNA probe-based PCR

In order to improve sensitivity and specificity of the real time assay, new primer sets were designed for their use in probe-based real time PCR (Table 2), using Universal Probe Library from Human catalogue (Roche, Germany). The combination of primers and a fluorescence probe can provide specific amplification and detection of the target sequence in a real time PCR assay, and thus, to avoid the inconvenient that SYBR-Green chemistry showed. Efficiency, sensitivity and specificity were tested by LNA probe-based PCR with all Pis v primers. Standard curves were obtained from *T. spelta* flour spiked with known amounts of defatted pistachio flour, from 100,000 to 0.5 mg/kg, and were optimal according to ENGL criteria (slope between −3.1 and −3.6, PCR efficiency between 90% and 110%, and R2 above 0.98) (Fig. 1C supplementary material). Specificity of all Pis v primers was assayed with DNA from nuts and plants used on food industry. Finally, Pis v 1 was selected as target, with the best results for specificity and sensibility, since the rest of Pis v primers were unsuitable of differentiating between pistachio

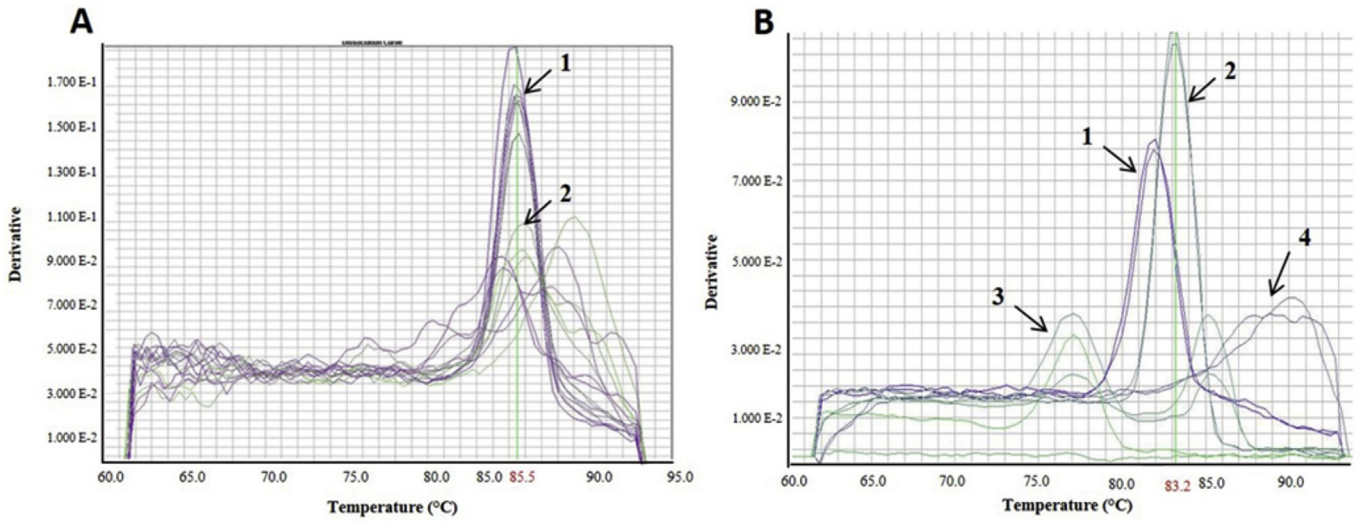

Fig. 1. A) Melting curve of amplification with SYBR-Green dye target Pis v 1 gene, from (1) mixture containing 100000 to 1 mg/kg of pistachio flour and (2) *T. spelta* wheat. B) Melting curve of amplification with SYBR-Green dye target Pis v 2 gene, of (1) mixture containing 10% of pistachio flour, (2) cashew, (3) orange and (4) *T. spelta* wheat. NTC was not detected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and citric fruits (Pis v 3, Pis v 5), peanut (Pis v 2 and Pis v 5) or other Anacardiaceae (Pis v 2, Pis v 3) (Fig. 1D supplementary material).

Standard curves from other *P. vera* varieties (Larnaka, Sirora, Aegina and Mateur), were also tested by probe-based real time PCR with Pis v 1 primers, obtaining acceptable efficiencies. The utilisation of this method allows the detection of different varieties of *P. vera*, with Ct values from 22 to 26 with 25 ng of DNA (Fig. 2 supplementary material). Other *Pistacia* species were not included in this work because of their lack of edible interest (Kafkas & Perl-Treves, 2002).

3.3. Comparison of both qPCR approaches

The comparative study was focused on Pis v 1 allergen coding sequence as selected target. Values of inclusivity and exclusivity of 100% were obtained for this primer pair (Brezna & Piknova, 2013, chap. 18). In order to determine the sensitivity, at least three different DNA isolations of wheat spiked with pistachio flour (from 100,000 to 0.05 mg/kg) and four technical replicates were evaluated.

Adequate efficiency values and sensitivity, as well as slope and linearity (R^2), were obtained for Pis v 1 primers and its probe. PCR method based on SYBR-Green chemistry resulted unable to distinguish the target from the wheat matrix when the pistachio quantity in the mixture was below 100 mg/kg, as described before. As a consequence, limit of detection (LOD) and limit of quantification (LOQ) were determined to be 100 mg/kg0 (or 01% w/w) and 1000 mg/kg (0.1% w/w) respectively.

The limit of detection is considered as the lowest concentration with positive identification of the target at least in 95% of the cases (Bustin et al., 2009). LOD for LNA probe-based real time PCR was 10 mg/kg (0.001% w/w), resulting more sensitive than SYBR-Green. The limit of quantification (LOQ) was also determined in 10 mg/kg of pistachio (Fig. 2). Moreover, with LNA probe-based real time PCR, could be possible to detect pistachio when the quantity in the mixture was 1 mg/kg, in 11 out of 14 replicates (more than 75% of positive amplification).

To assess the specificity of Pis v 1 primers, up to 50 ng of DNA from other nuts and plants species, commonly used as food ingredients (peanut, walnut, almond, cashew, mango, orange, lemon, wheat, rye, peach), was analysed by SYBR-Green and LNA probe-based PCR. Using SYBR-Green chemistry, we obtained Ct values below cycle 34 with DNA from some species as cashew, almond, mango, orange and lemon that could be considered as positive results, in contrast to LNA probe-based assay, with which all the results are negatives. Accordingly, using Pis v 1 as a target, real time based on SYBR-Green chemistry was a method less specific than probe-based one.

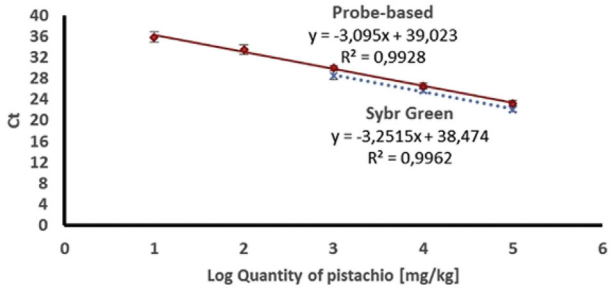

Fig. 2. Comparison of Pis v 1 standard curves obtained with probe-based and SYBR-Green-based real time PCR of mixtures containing different amounts of pistachio flour in wheat. Mean values and standard deviations of N = 18 (in probe-based) and N = 10 (in SYBR-Green) is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As a conclusion, the best results of sensitivity and specificity were obtained with Pis v 1 primers and probe-based real time PCR. The selection of a short amplicon for Pis v 1 (81 pb) is essential for the analysis of food products, normally submitted to thermal processing (with and without pressure) which can lead to DNA fragmentation (Hird et al., 2006). This combination of primers, probe and detection chemistry was selected to study the thermal processing effect on the LOD and to evaluate the convenience of this system on food products.

3.4. Effect of boiling and autoclave treatments

The influence of different treatments on the allergenic properties of food allergens has been thoroughly investigated (Sathe, Teuber, & Roux, 2005; Somkuti & Smeller, 2013), obtaining different results depending on the process and the analysed

sample. The effect of thermal processing on tree nuts allergenicity has been studied in walnut (Cabanillas et al., 2014), peanut (Cabanillas et al., 2015), hazelnut (Prieto, Burbano, et al., 2014b), cashew (Mattison et al., 2016) and even pistachio (Noorbakhsh et al., 2010b), observing a reduction of allergenicity after steam-roast treatment. The method of steam-roasting process, as practiced in that study, appears to reduce the IgE-binding of pistachio nut to a greater extent than does the method of dry roasting, a practice widely used in the pistachio industry worldwide. The results showed protein alterations might be occurring as a result of ionic strengths of soaking solution and heat processing conditions applied for steam-roasted pistachio nut. These protein alterations might be responsible for the reduction in IgE-binding. Commercially, ELISA tests are the most commonly used assay for detecting allergenic ingredients in foodstuff. PCR methods have been proposed as a powerful alternative to this protein-based test, due to the higher stability of DNA compared to protein molecules after heat treatments (Prado et al., 2015). In spite of that, processing may provoke the degradation of DNA molecules in different degree, and real time PCR as allergen detection method should be able to reach suitable sensitivity, not only in raw samples, but also in treated ones, commonly subjected to processing as heat and pressure (Costa et al., 2013; Linacero et al., 2016; Prieto et al., 2014a). Many studies about the effect of heat and pressure in detection by real time PCR have been published, establishing a significant increase in the Ct for the detection of autoclaved turkey samples by real time PCR, due to DNA fragmentation, whereas no effect was observed with the just boiled material (Hird et al., 2006). In our work, probe-based real time PCR has been selected for determining the effect of boiling and autoclave treatments, both of them commonly used in food industry, over the amplification of Pis v 1 target. Three DNA extractions for each treatment and spiked level (from 10% to 0.01%) were analysed in duplicate. Firstly DNA obtained from spiked samples (raw and treated) was tested using 18S universal primers and probe, by real time PCR, confirming that the quality and purity of DNA were adequate for PCR amplification (data not shown). A calibration curve has been then performed for each treatment, by plotting the average Ct values against the quantity of pistachio flour in the spiked sample. NTC (non template control) samples were always not detected.

Boiling treatment did not affect the amplification of Pis v 1 target, although the detection was retarded a cycle approximately in all the spiked samples. Efficiency, slope and correlation coefficient values indicated a high performance (Table 2 supplementary material). Thermal treatment on pistachio nuts, applied with pressure, reduced drastically the ability to amplify the target (Fig. 3B). In the case of boiling for 30 min, all the points were in the calibration curve. Sensitivity was gradually reduced with temperature (121 °C or 138 °C) and time (15 min or 30 min), demonstrating that this treatment affected pistachio amplification and detection by probe-based real time PCR (Fig. 3A). The softest autoclave treatment (121 °C for 15 min) maintained the linearity until 1000 mg/kg (0.1%), obtaining adequate efficiency and slope values of the standard curve. Sensitivity of real time PCR is reduced 100X after 15 min at 121 °C compared to control, and pistachio detection was only possible in 2 out of 4 replicates in mixtures with 100 and 10 mg/kg in these conditions. In the harshest process (138 °C for 30 min), pistachio DNA was amplified only in half of the replicates (Table 2 supplementary material). DNA degradation produced by the heat treatment with pressure reduced the capability of detect and amplify pistachio by real time PCR properly, as we observed after autoclave treatment in walnut (Linacero et al., 2016). Under increased pressure, DNA integrity is affected strongly and the DNA fragment size could be not long enough to amplify suitably, as has been reported in GMO detection (Ballari & Martin, 2013). By contrast, the size of the DNA fragments after boiling treatment is probably over the amplicon size (81 bp), and as a result, this processing has no effect on pistachio amplification. Previously, our group has demonstrated that high hydrostatic pressure treatment applied on hazelnut (Iniesto et al., 2013) and walnut (Linacero et al., 2016) did not affect DNA amplification, using SYBR-Green-based real time PCR. The method described in this work and based on LNA probes allowed to detect Pis v 1 sequence under harsh conditions (heat under pressure), being the amount of pistachio in mixtures quantifiable until 15 min at 121 °C.

3.5. Applicability on commercial food products

The applicability of LNA probe-based real time PCR using Pis v 1 primers for determining pistachio presence was assayed by analysing of 14 commercial food products, including cookies, chocolate, meat, sauce and snacks. In three of these foods the presence of pistachio is indicated in their label, eight of them adverted that the product could have traces of tree nuts and the rest did not declare any nut content. All food samples were amplified by this method with 18S primers as false negative control, resulting some of them in a high Ct value due to the DNA fragmentation presented in harsh processed foods.

ELISA was also carried out with food products in order to compare both results (Table 3). The correspondence between real time PCR with Pis v 1 primers and ELISA was 12 out of 14 food products analysed. Pistachio ELISA kit (LOQ 1–40 mg/kg) detected two false positives, pesto sauce, which contains 5% of cashew nut, and chocolate with hazelnut, with which the ELISA test showed cross-reactivity.

With this real time PCR assay, we could estimate the pistachio content in mg/kg, present in a food product, by substitution in the obtained Ct in the standard curve. For food samples with pistachio declaration, the estimated amount of pistachio is exactly 5% in chocolate with pistachio but slightly lower for cold meat (approximately 0.5% instead of 1% reported in the label). This could occur because of the presence of different type and amount components that might interfere with the reaction (Lopez-Calleja et al., 2014). A different standard curve for any kind of matrix, with the same processing and composition than the food sample would be necessary for a real quantitative assay, and it is unviable from a practical point of view.

The method developed in this work in order to detect pistachio allergen coding sequence by LNA probe-based real time PCR is more specific, reliable and sensitive compared to SYBR-Green chemistry. It is also accurate and specific for the detection of small amounts of pistachio in processed commercial food products. Moreover, the findings from processed pistachio samples are interesting, because of the scarce of studies focused on food processing over pistachio, and boiling and autoclave in particular. Further studies about the effect of food processing treatments over real time PCR performance and allergens detection should be carried out, in order to develop techniques capable of detecting traces of allergens in food and ensure the consumer safety.

4. Conclusions

In this work, our group has compared two well-known approaches of real time PCR, based on SYBR-Green chemistry and the use of LNA probes, to detect pistachio traces in food products and processed samples. According to our results, probe-based real time PCR seems to be more reliable, sensitive and specific for detecting Pis v 1 allergen coding sequence than SYBR-Green chemistry. This assay is able to detect up to 10 mg/kg of pistachio in complex mixtures and its applicability has been demonstrated in

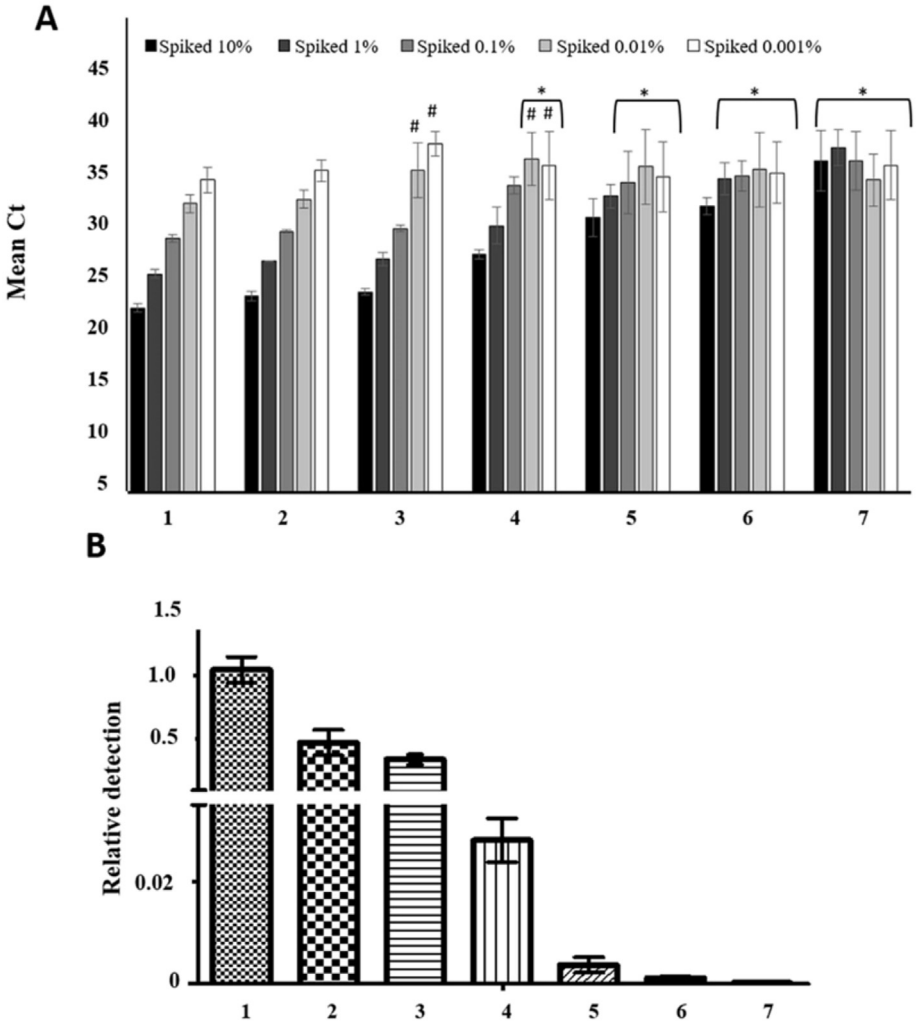

Fig. 3. A) Amplification of Pis v 1 target in spiked samples from untreated (1) and treated pistachio nuts: boiling 30 min (2), boiling 60 min (3), autoclave 121 °C 15 min (4) and 30 min (5), autoclave 138 °C 15 min (6) and 30 min (7). B) Relative detection of the Pis v 1target in the mixture with 10% of pistachio flour from untreated (1) and treated samples (2–7). Mean and its standard deviation are represented. *No significant differences among Ct values in different spiked levels within the same treatment, by ANOVA analysis. #Ct values out of the calibration curve.

Table 3
Pis v 1 detection by probe-based real time PCR analysis on commercial food products. At least three different DNA isolations and two technical replicate was tested.

Commercial food	Label declaration	Pis v 1[a]	18S rRNA[a]	ELISA
Cereal bar	Almond	39.46 ± 0.39	17.57 ± 0.46	−
Cereal bar II	Might contain nuts	N.D.	22.35 ± 2.25	−
Chocolate with hazelnut	Hazelnut	38.34 ± 1.14	18.42 ± 1.67	+
Cereal bar with hazelnut	Might contain nuts	38.55 ± 0.74	17.78 ± 0.38	−
Black chocolate	Might contain nuts	N.D.	18.73 ± 0.03	−
Cookies with cereal and chocolate	Might contain nuts	38.59 ± 0.54	20.17 ± 1.05	−
Vegetal burger	Might contain nuts	39.38 ± 0.39	15.32 ± 0.26	−
Pesto sauce	5% cashew	37.80 ± 0.87	14.73 ± 0.10	+
Cookies with fiber	Nuts not declared	38.05 ± 0.83	24.92 ± 0.63	−
Chocolate bar	Nuts not declared	38.48 ± 0.81	20.71 ± 1.50	−
Cookies with chocolate	Nuts not declared	38.84 ± 0.61	20.43 ± 0.49	−
Chocolate with pistachio	5% pistachio	24.44 ± 0.79	12.06 ± 0.20	+
Cold meat with pistachio	1% pistachio	28.15 ± 0.28	19.99 ± 0.88	+
Jam with pistachio	Pistachio[b]	26.93 ± 0.36	18.31 ± 0.68	+

ND indicates no positive signal after 40 cycles of PCR in any replicate.
[a] Average Cycle threshold (Ct) ± standard error.
[b] Nut amount not reported/declared.

commercial foodstuff, comparing the outcomes with ELISA kit. The use of this strategy allowed to detect pistachio even in processed samples by boiling and autoclave, being possible to quantify the pistachio amount in the softest treatments.

Note

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2016.12.015.

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Appendix 5. Supplementary Material

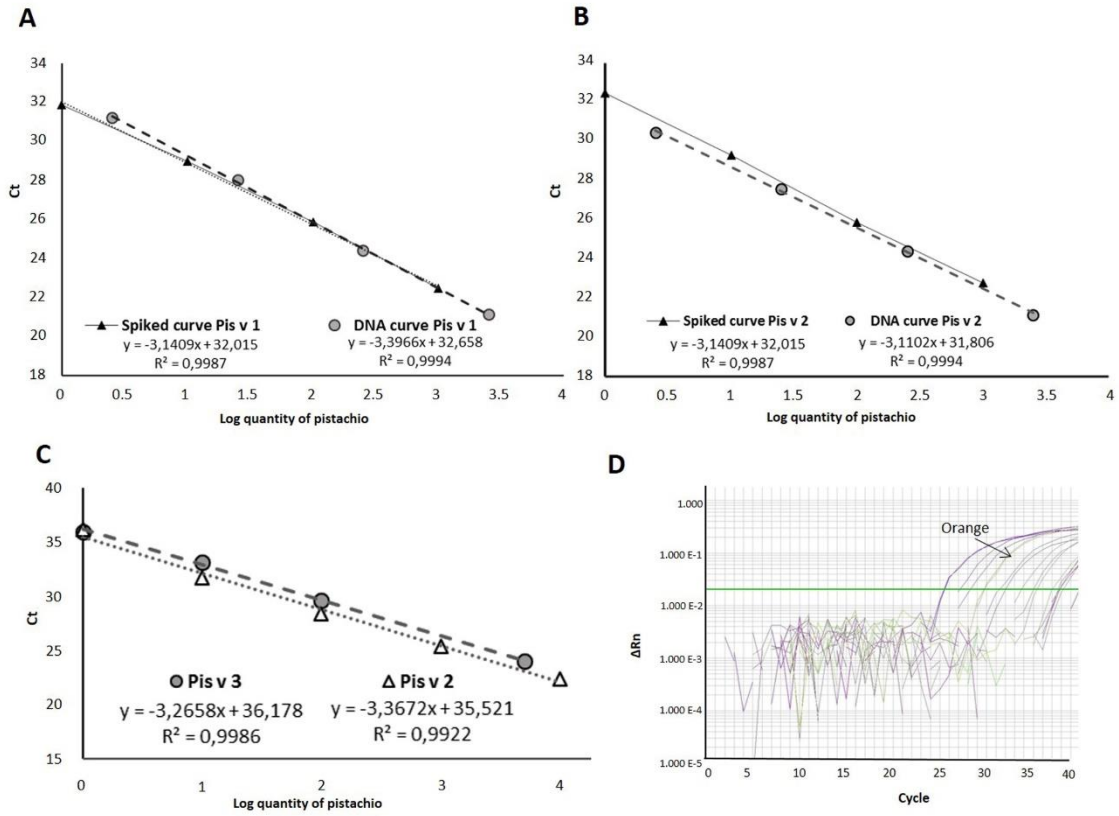

Figure 1 Supplementary material. Comparison of standard curves from pistachio DNA or mixtures containing different amounts of pistachio flour in wheat (spiked) with different primer sets as (A) Pis v 1, efficiency 92% for DNA and 108% for spiked curve and (B) Pis v 2, efficiency 108% for DNA and 103% for spiked curve, by SYBR-Green. (C) Standard curves from wheat mixtures containing different amounts of pistachio flour for Pis v 2 and Pis v 3 and (D) plot of amplification of Pis v 5 primer set of spiked curve and some DNA from different species LNA-based real time PCR. Orange, hazelnut and cashew plots amplification are marked by an arrow in the graphic.

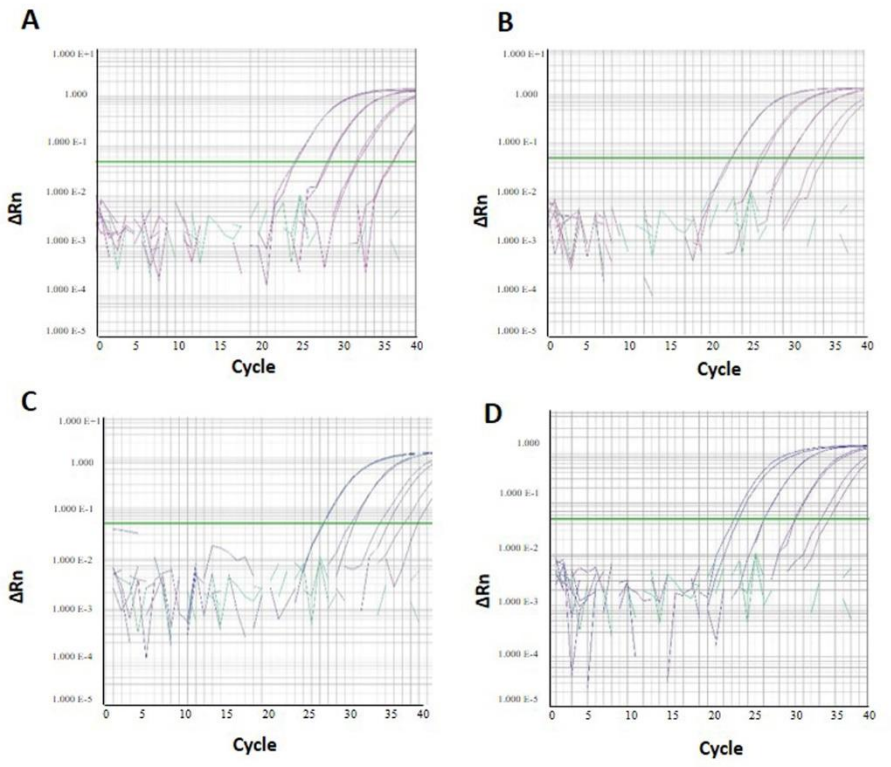

Figure 2 Supplementary material. Plots of amplification of the standard DNA curves from the four pistachio varieties analysed by probe-based real time PCR using Pis v 1 primers. P.vera (A) var. Sirora, (B) var. Mateur, (C) var. Larnaka and (D) var. Aegina.

Table 1 Supplementary material. Expected and observed amplicon size for each pistachio allergen coding sequence.

Sequence	Accesion number	Sequence (5´→ 3´)	Expected amplicon (bp)	Observed amplicon (bp)
Pis v 1	DQ631675.1	GTCCGTACTTCTCCTATCTGCC ATGGTGAACTGAACTGACACCC	428	428
Pis v 2	DQ631677.1	GGAGTCAGCAATCAGGAGAACA GAGGATGGGAAGGTTTAAGGCA	569	730
Pis v 3	EF116865.1	AGAGGAAGAAGAGGGAATGGGG TACTTCCCTGCTGAAAGCTCTG	429	855
Pis v 4	EF470980.1	AGGAAAACCCTACAAGAATCCC AAGCTTCTTCGACTCCTTGTCC	474	1580
Pis v 5	EU410073.1	ACGGACAGAGTAGTAGGTTCCA TCTGGGGTGTAAATGTCAGAGC	577	691

Table 2 Supplementary material. Detection of Pis v 1 target by probe-based real time PCR in untreated (control) and treated spiked samples.

Pistachio amount (mg/kg)		**Boiling treatments**	
	Control[1]	Boiling 30 min	Boiling 60 min
100000	23.14 ± 0.11 (12/12)[2]	24.28 ± 0.23 (4/4)	24.73 ± 0.14 (6/6)
10000	26.49 ± 0.12 (12/12)	27.70 ± 0.03 (4/4)	27.93 ± 0.26 (6/6)
1000	29.95 ± 0.11 (12/12)	30.65 ± 0.07 (4/4)	30.93 ± 0.12 (6/6)
100	33.26 ± 0.26 (12/12)	33.81 ± 0.45 (4/4)	36.63 ± 1.97 (4/6)
10	35.69 ± 0.44 (8/8)	36.60 ± 0.61 (3/3)	39.22 ± 0.49 (2/6)
Slope	-3.18	-3.18	-3.09
Efficiency (%)	105.96	105.95	110.30
R^2	0.9962	0.9995	0.9855

Pistachio amount (mg/kg)	**Autoclave treatments**			
	121ºC 15 min	121ºC 30 min	138ºC 15 min	138ºC 30 min
100000	28.36 ± 0.19 (6/6)	31.98 ± 0.75 (6/6)	33.08 ± 0.34 (6/6)	37.55 ± 1.20 (3/6)
10000	31.22 ± 0.74 (6/6)	34.12 ± 0.47 (6/6)	35.79 ± 0.62(6/6)	38.88 ± 0.71 (2/6)
1000	35.14 ± 0.41 (4/4)	35.48 ±1.24 (5/6)	36.11 ± 0.59 (6/6)	37.56 ± 1.16 (3/6)
100	37.77 ± 1.31 (2/4)	37.00 ± 1.49 (3/6)	36.69 ± 1.52 (3/6)	35.68 ± 1.04 (5/6)
10	37.10 ± 1.68 (2/4)*	36.00 ± 1.39 (4/6)	36.39 ± 1.22 (4/6)	37.12 ± 1.38 (3/6)
Slope	-3.24			
Efficiency (%)	110.32			
R^2	0.9934	0.7902		

[1]Ct±SE

[2]Positive amplifications/Total replicates

*Detection is possible but Ct is not in the calibration curve.

Artículo 6

EVALUACIÓN DE LAS SONDAS DE ÁCIDO NUCLEICO BLOQUEADO Y TAQMAN PARA LA DETECCIÓN ESPECÍFICA POR PCR EN TIEMPO REAL DE ANACARDO EN ALIMENTOS PROCESADOS.

El fruto del anacardo (*Anacardium occidentale*) puede desencadenar reacciones severas en pacientes alérgicos, incluyendo anafilaxis y muerte. El etiquetado de la presencia de anacardo en alimentos es obligatorio y en consecuencia, deben desarrollarse métodos analíticos sensibles y específicos para su detección. En este estudio, se han analizado las secuencias codificantes de Ana o en distintas variedades de anacardo. Se han utilizado dos tipos de sondas de hidrólisis para la diana Ana o 1, LNA y TaqMan, comparándose su eficiencia, sensibilidad, límite de detección y especificidad. Se han desarrollado ensayos precisos de PCR en tiempo real para detectar y cuantificar hasta 10 ppm de anacardo en mezclas complejas. Además, se ha analizado la influencia de la cocción y el tratamiento de autoclave sobre la detectabilidad de anacardo, observándose que con ambas sondas se obtienen los mismos resultados. Este método analítico fue capaz de detectar hasta 1000 ppm de anacardo tratado por autoclave mientras que la cocción no afectó a su detección. Se ha estudiado la aplicación de este ensayo analizando varios alimentos comerciales y se han comparado los resultados con los obtenidos a través de un kit ELISA comercial, concluyendo que ambos tipos de sondas de hidrólisis son adecuadas para la detección de la diana Ana o 1.

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Evaluation of locked nucleic acid and TaqMan probes for specific detection of cashew nut in processed food by real time PCR

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ABSTRACT

Cashew (*Anacardium occidentale*) nut can trigger serious reactions in allergic patients, including anaphylaxis and death. Labelling the presence of cashew nuts in food products is mandatory and consequently, sensitive and specific analytical methods must be developed. In this study, Ana o allergen coding sequences have been sequenced in several cashew varieties. Two hydrolysis probes, locked nucleic acid (LNA) and TaqMan, have been designed and their efficiency, sensitivity, limit of detection and specificity for Ana o 1 coding-sequence detection have been compared. Reliable Real Time PCR assays to detect and quantify up to 10 ppm of cashew nuts in complex mixtures have been developed. Moreover, the influence of boiling and autoclave treatment on cashew nut detectability has been analysed by qPCR, showing both probes similar performance. This analytical method was able to detect up to 1000 ppm with good functionality in autoclave treated samples. Boiling did not affect cashew nut detectability. Both hydrolysis probes are suitable for Ana o 1 coding sequence detection. Applicability of the assay has been studied by analysing several food products, and comparing the results with those of a commercial ELISA kit.

1. Introduction

Tree nuts are valuable foods rich in proteins, minerals, vitamins, antioxidants and a considerable high content of unsaturated fatty acids (Ros, 2010), and their global production and consumption is increasing in the last years. Tree nuts allergy has also increased, although prevalence varies among individual nut and regions and it is difficult to stablish. In Europe, even though the most common allergies to tree nuts correspond to hazelnut and walnut, cashew nut allergy is getting importance over the last years according to several reports, with a variable prevalence among countries (Mendes, Costa, Vicente, Oliveira, & Mafra, 2016). Other studies indicate that allergies to walnut and cashew nuts are the most prevalent among tree nuts in the USA, achieving 20–30% and 15–30%, respectively (McWilliam et al., 2015). Originally from Brazil, cashew tree is cultivated in Africa and Asia, and mainly produced in India, and its fruits, cashews, are appreciated worldwide (Mendes et al., 2016). Cashew nuts are commonly consumed as snack or as ingredient in biscuits, sauces, sweets and several food dishes, and medical efficiency of cashew nuts has also been recently studied (Mah et al., 2017). Until the date, three major allergens have been identified, all of them being seed storage proteins (WHO-IUIS Allergen Nomenclature Sub-Comittee): Ana o 1, a 7S vicilin (Wang et al., 2002), Ana o 2, a 11S legumin (Wang, Robotham, Teuber, Sathe, & Roux, 2003), and Ana o 3, a 2S albumin (Robotham et al., 2005).

In order to protect the safety of the allergic patients, European regulation obliges to advise the presence of tree nuts in food labels (Official Journal of the European Union (22/11/2011), 2011 Regulation (EU) No 1169/2011). The presence of allergenic ingredients in foods can be either a consequence of fraudulent substitution or adventitious contamination in the food facility. Last option is more likely for cashew nuts because of its high price. There is no treatment for food allergies, and sensitized individuals have to totally

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avoid the consumption of offending ingredients. Therefore, a reliable and specific tool for detecting traces of specific food allergens is indeed essential to improve the quality of life of sensitized individuals. Enzyme-linked immunosorbent assay (ELISA) is the common method for detecting small amounts of proteins from specific foods, as cashew nuts, and it is possible to find several ELISA tests on the market. One of the disadvantages of this technique is the possible effect of food processing on protein solubility, because the subsequent detection with protein-based techniques might be altered (Mattison et al., 2016). In the last years, DNA-based methodologies, such as Real Time PCR, microarrays and also DNA biosensors, have been proposed as specific, sensitive and reliable alternatives to ELISA (Sun, Guan, Shan, Zhang, & Li, 2012), since DNA molecules can preserve its integrity better than proteins. Several studies have been carried out to develop Real Time PCR methods for detecting allergens in foods (Costa, Fernandes, Villa, Oliveira, & Mafra, 2017; Prado et al., 2016), cashew nuts included (López-Calleja, Cruz, González, García, & Martín, 2015a; Piknová & Kuchta, 2007), with different approaches and chemistries, and obtaining promising results.

The selection of the specific target and chemistry are essential parts of a new Real Time PCR experiment, because specificity, sensitivity and potential of quantification of the method directly depend on them. Some researchers have used multi copy genes such as mitochondrial, chloroplast or repetitive sequences as Internal Transcriber Sequences (ITS), to detect an allergenic ingredient in food (Demmel, Hupfer, Hampe, Busch, & Engel, 2008; Lopez-Calleja et al., 2013), while others have published detection methods using allergen coding sequences as targets, e.g. Ara h 2 in peanut (Hird, Lloyd, Goodier, Brown, & Reece, 2003), Cor a 9, 11 and 13 in hazelnut (Iniesto et al., 2013), Jug r 1 and 3 in walnut (Linacero et al., 2016), Pis v 1 (Sanchiz et al., 2017) or Ana o 3 in cashew nuts (Brzezinski, 2006; Piknová & Kuchta, 2007), among others.

On the other hand, Real Time PCR assay can be performed using several chemistries commercially available: intercalating dyes, primer-based or probe-based chemistries. However, very few studies have been published addressing a practical comparison among different chemistries for application to the detection of GMO or allergen traces (Buh Gašparič et al., 2010).

Locked Nucleic Acid (LNA) and classical Taqman probes are both hydrolysis probes, also called 5′-nuclease probes, commonly located between the PCR primers as fluorescence oligonucleotides. Thus, high specificity can be achieved compared to other alternatives such as intercalating dyes. LNA are modified DNA analogues primers with high melting temperature that allows them to be shorter and highly specific when compared to classical TaqMan probes. Both probes carry a fluorophore attached to one extreme and a quencher attached to the other; when they are physically close, the quencher prevents fluorescence. Once the probe is cleaved by the DNA polymerase, the reporter emits fluorescence (Valasek & Repa, 2005).

Tree nuts, as other foods, usually undergo thermal treatment to improve organoleptic properties, ensure food safety and sometimes modify allergenic properties. Recently, the influence of enzymatic hydrolysis and thermal treatment on cashew nut allergens have been studied (Cuadrado et al., 2018; Sanchiz et al., 2018). Fragmentation and/or degradation of DNA molecules have been reported by several authors after severe treatments (Costa, Oliveira, & Mafra, 2013; Gryson, 2010; López-Andreo, Aldeguer, Guillén, Gabaldón, & Puyet, 2012) and some studies have been performed in order to analyse the effect of processing, such as thermal treatment with and without applying pressure (boiling, High Hydrostatic Pressure HHP, autoclave, frying, roasting), on the detection of different DNA targets, in peanut, hazelnut, walnut, almond and pistachio (Iniesto et al., 2013; Linacero et al., 2016; Prieto et al., 2014; Sanchiz et al., 2017; Scaravelli, Brohée, Marchelli, & Van Hengel, 2009).

The present study is aimed to set up a reliable and suitable real time PCR-based detection assay for Ana o 1 allergen coding sequence from cashew nuts using two different hydrolysis probes, Locked Nucleic Acid and classical TaqMan, which have been compared regarding efficiency, sensitivity, specificity and applicability in several commercial food products. Comparison with a commercial ELISA kit has also been performed. We particularly focused the analysis on the influence of six different treatments, based on boiling and autoclave, on the detectability of cashew nut target Ana o 1 by Real Time PCR.

2. Materials and methods

2.1. Samples

Five varieties of raw *Anacardium occidentale* L. (CCP006, 1001, BRS189, BRS274, Embrapa 50) nuts were analysed in this work, provided by Germplasm Bank of Embrapa, Brazil. A commercial ready-to-eat variety of cashew nut from India (type 320) was provided by Productos Manzanares S.L. (Cuenca, Spain). Other plant species commonly found as food ingredients were also used as well as food products which were purchased in local stores, ground with a kitchen robot (Thermomix 31-1, Vorwerk Elektrowerke, GmbH & Co. KG, Wüppertal, Germany) and stored at −20 °C.

Several points of binary mixtures (0.5, 1, 5, 10, 100, 1000, 10000, 100000 mg/kg) were performed by mixing known amounts of defatted cashew nut flour in spelt wheat (*Triticum spelta* L.) flour, in a final weight of 100 g. The mixture containing 10% cashew nuts (100.000 mg/kg) was prepared by adding 10 g of cashew nut flour to 90 g of spelt wheat flour, and followed by 10 or 5-fold dilutions to 0.5 mg/kg, all of them being homogenized with the kitchen robot.

2.2. Thermal and pressure treatments

One hundred grams of cashew nuts type 320 were boiled in distilled water (1:5 w/v) for 30 or 60 min, or autoclaved using a Compact 40 Benchtop autoclave (Priorclave, London, UK) at 121 °C (120 kPa) or at 138 °C (256 kPa) for 15 or 30 min. Untreated and treated cashew nuts were freeze-dried (Telstar Cryodos frezze-dryer), ground using a Cyclotec 1093 Sample Mill with a sieve of 1 mm, defatted with n-hexane for 4 h (34 ml/g of flour) and air-dried. Binary mixtures in spelt wheat flour were performed as described before with defatted treated cashew nut flours, containing 10, 100, 1000, 10000 and to 100000 mg/kg (0.01%–10% cashew nuts). All samples were stored at 4 °C.

2.3. DNA extraction

One hundred milligrams of cashew nuts from the six varieties were extracted using i-Genomic Plant DNA Extraction Mini Kit (iNtRON biotechnology), following the manufacturer instructions. At the same time, 100 mg of each binary mixture were homogenized in 1000 μl of Cetyl trimethylammonium bromide (CTAB) with 1% of polyvinylpyrrolidone (PVP) and 3 μl of 25 mg/ml RNAse, and were incubated at 65 °C for 30 min, shaking every 10 min. Then, 800 μl of chloroform were added to the sample before centrifugation at 13000 rpm for 10 min. Genomic DNA was obtained from 800 μl of aqueous supernatant, which were processed with the Power Plant DNA isolation kit (MoBio, CA USA) and finally eluted in 100 μL of Tris HCl pH 8.3.

Two hundred milligrams of each food sample were homogenized in liquid nitrogen and genomic DNA was obtained using the NucleoSpin kit (Macherey-Nagel, Düren, Germany) following the

manufacturer instructions, with some modifications for chocolate-based products (Sanchiz et al., 2017).

Quality of isolated DNA was evaluated on 0.8% TBE agarose gels and both DNA quality and concentration were determined spectrophotometrically with NanoDrop ND-1000 spectrophotometer (Thermo-Fisher, Waltham, MA, USA), measuring absorbance at 230, 260 and 280 nm.

2.4. Conventional PCR, cloning and sequencing

Primers used in this work were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa, USA) or Metabion (Metabion International, Planegg/Steinkirchen, Germany).

Primers for conventional PCRs were designed with NCBI Primer-Blast program on the target allergens coding sequences of Ana o 1 (Genebank accession no. AF395894.1, Ana o 1 fw GCCATAATGGGTCCGCCTAC; Ana o 1 rev GGTGGTGAAATCTTCGTCTTCG), Ana o 2 (Genebank accession no. AF453947.1, Ana o 2 fw TTACCAAGCGCCCCAACAGG; Ana o 2 rev GATGGGCGGATCACCCGAAG) and Ana o 3 (Genebank accession no. AY081853.1, Ana o 3 fw TATCTGCCTTCGCAGTCCTCC; Ana o 3 rev TTCCCGAACCTCCTCACCCTTT).

Conventional PCR reactions were performed with DNA from the cashew nut varieties CCP06, 1001, BRS189, BRS274, Embrapa 50 and the commercial type 320. These reactions were carried out in 20 μl, containing 25 ng of DNA, 0.25 μM of each primer and 1XFastStar PCR Master Mix (Biotools, Loganholme, Australia). SensoQuest LabCycler (Progen Scientific Ltd, Germany) was programmed with an initial denaturation step at 95 °C, 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C, 1 min and elongation at 72 °C for 1 min, and a last step at 72 °C for 6 min. The resulted amplicon of Ana o 1 was sequenced in an ABI PRISM 3700 sequencer (Applied Biosystems, Foster City, CA, USA) in the "Servicio de Genómica (Universidad Complutense de Madrid)", whereas amplicons obtained with Ana o 2 and Ana o 3 primers were cloned in TOPO TA pCR4 Cloning kit (Invitrogen, Inc., UK) following the manufacturer's instructions. Plasmid DNA was purified using Plasmid DNA purification kit (Biotools, Loganholme, Australia) and sequenced as described before. Software Bioedit (Ibis Biosciences, Carlsbad, CA, USA) was employed to edit DNA sequences. Sequences were compared with those in the GenBank (NCBI) nonredundant nucleotide (nr/nt) databases, through BLAST, and alignments between sequences of the allergen genes from the six varieties and homologue sequences from other plant species were performed using ClustalW algorithm (Thompson, Higgins, & Gibson, 1994).

2.5. Real time PCR

The conserved sequences between cashew nut varieties for each target (Ana o 1, Ana o 2 and Ana o 3) were used to design specific primer pairs and LNA probes using the Universal Probe Library (Roche, Germany). Moreover, TaqMan probe for Ana o 1 target was also designed in order to compare both hydrolysis probes using the same primers in Real Time PCR (Fig. 1). Primers fit requirements related to their length and amplicon size (Table 1).

Real Time PCR reactions were performed with 7900HT Fast Real-Time PCR (Applied Biosystems, CA, USA). Different final probe or primer concentration were tested to find the proper functioning conditions. Probe-based Real Time PCR were performed in a volume of 20 μl with 10 μl of TaqMan®Gene Expression Master Mix (Applied Biosystem, CA, USA), 0.25 μM of each primer, 0.15 μM of Ana o 1 TaqMan probe or 0.1 μM of LNA probe, and 5 μl of extracted DNA at different concentrations. Running conditions included an initial denaturation step at 95 °C, for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and primer annealing and elongation at 60 °C for 1 min. At least two experimental and three technical replicates were analysed, and two non-template controls (NTC) were included in each plate to check the PCR performance.

The cycle threshold (Ct) value, obtained from 10-fold serial dilutions of cashew nut genomic DNA and from binary mixtures of untreated cashew nuts, were used to generate standard curves for Real-Time PCR. The efficiency ($10^{(-1/slope)}$-1) of each reaction was calculated from the slope of each standard curve (Ct vs log DNA content or Ct vs log Quantity of cashew nut flour).

The effect of thermal processing over PCR amplification, whether applying pressure or boiling, were analysed and compared using binary mixture samples of processed cashew nut flour in spelt wheat (from 10 to 100000 mg/kg), by detecting Ana o 1 with both probes. Applicability of this assay was tested by the final evaluation of cashew nut presence in 14 commercial foodstuffs. To avoid false negative results, all extracted DNA were evaluated by LNA probe-based Real Time PCR or conventional PCR using 18S primers, listed in Table 1, as positive amplification control.

2.6. ELISA assay

Cashew nut proteins were detected by immunochemistry in several commercial food products with a commercial ELISA kit according to the manufacturer's instructions. The kit included microtiter plates pre-coated with anti-cashew antibodies and the quantification range was from 2 ppm to 60 ppm (AgraQuant cashew assay, Romer Labs, UK).

2.7. Statistical analysis

Linear regression analysis was performed to test differences between slopes of calibration curves from binary mixtures of untreated and treated samples (boiling or autoclave) for both Real Time PCR systems, using GraphPad Prism Programme. Significant differences were considered when $p < 0.05$.

3. Results and discussion

3.1. Selection of the target

In this study, performance features of Real Time PCR using either

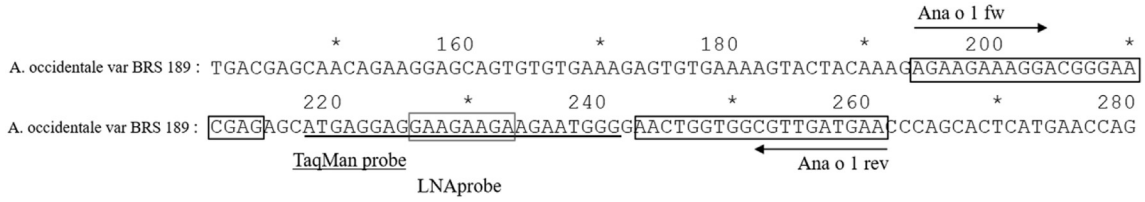

Fig. 1. Primers and LNA and TaqMan probes designed from Ana o 1 coding sequence in cashew nut. Sequence and position of primers and probes in Ana o 1 coding sequence from *Anacardium occidentale* variety BRS 189. Primers forward and reverse are included in black frames, LNA probe in grey frame and TaqMan probe is underlined.

Table 1
DNA sequences of primers and probes used for real time PCR experiments. The length of the resulting amplicon is also indicated.

Primers	Sequence (5′→ 3′)	Fragment length
Ana o 1 fw	AGAAAGGACGGGAACGAGAG	65
Ana o 1 rev	TTCATCAACGCCACCAGTT	
LNA probe*	5′-FAM-GGAGGAAG-Q-3′	
TaqMan probe	5′-FAM-ATGAGGAGGAAGAAGAAGAATGGG-BHQ1-3′	
Ana o 2 fw	TTTTCTGGCTTCGATACAGAGTT	65
Ana o 2 rev	AGCTGCTTTATGAGACGTTCG	
LNA probe*	5′-FAM-GGCTGAGG-Q-3′	
Ana o 3 fw	AAGAAAGCTTGAGGGAATGCT	68
Ana o 3 rev	AGCTGCCTCACCATTTGC	
LNA probe*	5′-FAM-GCAGGAAG-Q-3′	
18S fw	CGCGAGAAGTCCACTAAACC	64
18S rev	CCTACGGAAACCTTGTTACGA	
LNA probe*	5′-FAM-GGAAGGAG-Q-3′	

*Universal Probe Library, Roche.

of the hydrolysis probes, LNA or TaqMan, were compared regarding efficiency, sensitivity, specificity and applicability to commercial food products. The selected target was a single copy gene, allergen coding sequences, which showed constant and low copy number, being thus a good candidate to detect a specific ingredient for quantitative purposes (Prado, Boix, & Holst, 2012).

Ana o 1, Ana o 2 and Ana o 3 sequences were obtained from NCBI database to design primer pairs on exons of the allergen coding sequences in six cashew varieties. The expected size of the amplicons were obtained for allergen coding sequences Ana o 1 (7S vicilin) and Ana o 3 (2S albumin) in six different varieties of *A. occidentale*, whereas Ana o 2 (11S globulin) amplicon was longer than expected, indicating the presence of an intron. Intervarietal differences in such allergen sequences were not detected (Supplementary Material Fig. S1); therefore, possible false negative results due to polymorphism among different varieties are minimized (D'Andrea et al., 2011).

The obtained sequences for each Ana o gene were aligned with homologue sequences from other species that showed identity values over 70% at Genbank. Real Time PCR using specific primers and LNA probes from Ana o sequences, were first performed to select the target which showed the best results regarding efficiency and specificity. All Ana o primers and probes exhibited values of efficiency and slope within the acceptable criteria when cashew nut DNA curves were assayed (99.98% and −3.33 for Ana o 1; 103.11% and −3.42 for Ana o 2; 93.66% and −3.49 for Ana o 3) (Supplementary Material Fig. S2). However, Ana o 1 showed better specificity than Ana o 2 and Ana o 3 primers and probes, which also detected other species, such as species of citrics, other Anacardiaceae, pistachio or mango (Supplementary Material Fig. S3). Finally, Ana o 1 allergen coding sequence was selected as target for the comparison of the ability of the two kinds of hydrolysis probes, LNA and TaqMan, to detect traces of cashew nuts by Real Time PCR (Fig. 1).

3.2. Comparison of hydrolysis probes: LNA vs TaqMan

The comparison included their performance characteristics, specificity and sensitivity to detect the same target, Ana o 1 allergen coding sequence, using the same primers (Fig. 1). Efficiency of the Real Time PCR reaction was calculated from the slope of the curve ($Eff = 10^{(-1/slope)}$) obtained with cashew nut DNA serially diluted or with several points of the binary mixtures (0.5, 1, 5, 10, 100, 1000, 10000, 100000 mg/kg) performed by mixing known amounts of defatted cashew nut flour in spelt wheat flour, above described. A slope of −3.32 corresponds to a 100% efficient PCR reaction; R^2 describes the correlation between the logarithm of the amount of cashew nut DNA or cashew nut flour and the Ct in the standard curves.

As observed in Fig. 2, both LNA and TaqMan probes showed acceptable efficiency percentages (between 90 and 110%) according to ENGL criteria (European Network of GMO Laboratories, 2015), calculated from DNA standard curve slope (efficiency 91.87% and 96.25% for LNA-based and TaqMan-based Real Time PCR, respectively) as well as from food matrix curve slope, obtained from at least five different extractions of nine binary mixture levels (efficiency of 98.40% for LNA probe-based and 97.29% for TaqMan probe-based assay).

Regarding the sensitivity, both systems were able to detect and quantify up to 2.5 pg of cashew nut DNA of different varieties (Fig. 2A). The lowest amount of target that can be detected in at least 95% of the cases is considered the Limit of Detection (LOD) (Bustin et al., 2009). LOD and Limit of Quantification (LOQ) were established for both probes in 10 mg/kg or ppm; additionally, with TaqMan probe, detection and quantification of the target was possible also with 5 ppm of cashew nuts in the binary mixture, but only in 61% of the replicates (Fig. 2B). Using LNA probe, detection and quantification of 5 ppm of cashew nuts in mixtures was also possible but only in 37% of the replicates. Although not significant, data showed that classical TaqMan probe might be slightly more sensitive than LNA probe for detection of cashew nut traces by Real Time PCR.

To assess the specificity of Ana o 1 primers with LNA and TaqMan probes, 25 ng of DNA from different extractions of fruits, nuts and other plant species found in food products (almond, pistachio, walnut, hazelnut, pecan, peanut, mango, orange, lemon, wheat, apple, pear, pepper) were analysed by Real Time PCR (Table 2).

An average Ct above 38 was obtained in some replicates (6 out of 18) when almond DNA was analysed with the TaqMan probe designed to detect Ana o 1 target (Table 2). It should be considered that 25 ng is a rather large amount of pure DNA while the obtained Ct is considerably high, being also improbable to find such quantity of almond in processed foods without label declaration. In contrast, all species, excepting cashew nut, with an average Ct of 22.22 ± 0.41, were not detected using LNA probe.

Short hydrolysis probes (8–9 mer), as LNA probes from Universal probe library, have been demonstrated to be very specific (Universal Probe Library Probes (Instruction Manual); Roche Applied Science, 2017) since a single nucleotide modification or difference may cause instability. Moreover, LNA probes have been found to be more interesting because of the difficulties for the optimal design of classical TaqMan probes for Real Time PCR

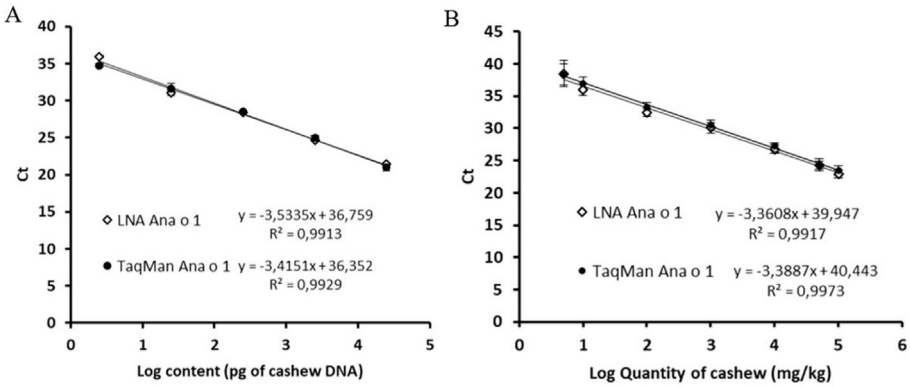

Fig. 2. Ana o 1 standard curves. Efficiency and sensitivity of LNA or TaqMan probe-based Real Time PCR to detect Ana o 1 target, with cashew nut variety 1001 DNA standard curve from 25 ng and serial dilutions up to 2.5 pg, (A) and standard curve from binary mixtures of spelt wheat flour containing different amounts of untreated cashew nut flour (B). Mean Ct values and standard deviations (at least n = 4 for DNA curve and n = 10 for binary mixtures) are shown.

Table 2
Species assayed with LNA and TaqMan probes in Ana o 1 real time PCR. Final concentration was 25 ng of DNA.

Common name	Specie	LNA probe[a]	TaqMan probe
Cashew	*Anacardium occidentale*	22.22 ± 0.41	23.48 ± 0.23
Almond	*Prunus dulcis*	N.D.	38.84 ± 0.50
Hazelnut	*Corylus avellana*	N.D.	N.D.
Walnut	*Juglans regia*	N.D.	N.D.
Pistachio	*Pistacia vera*	N.D.	N.D.
Pecan	*Carya illionensis*	N.D.	N.D.
Peanut	*Arachis hypogea*	N.D.	N.D.
Pepper	*Piper nigrum*	N.D.	N.D.
Wheat	*Triticum spelta*	N.D.	N.D.
Mango	*Mangifera indica*	N.D.	N.D.
Apple	*Malus domestica*	N.D.	N.D.
Lemon	*Citrus x limon*	N.D.	N.D.
Orange	*Citrus sinensis*	N.D.	N.D.
Pear	*Pyrus communis*	N.D.	N.D.

N.D. = not detected after 40 cycles of qPCR.
[a] Ct mean ± SE.

(length around 20–25, right melting temperature, suitable amplicon size) which is especially important for applications such as allergen detection in processed samples or GMO identification (Salvi, D'Orso, & Morelli, 2008).

3.3. Influence of moist thermal treatment in cashew nut detection

Cashew nuts are usually consumed as fried or roasted snack, but it is often incorporated as ingredient in processed food products such as sauces, cookies, chocolate, cakes, beverages, etc. Treated cashew nuts might also trigger allergenic reactions in very sensitized patients (Cuadrado et al., 2018; Sanchiz et al., 2018; Venkatachalam et al., 2008). Valuable results have been obtained by some groups regarding detection of untreated cashew nuts by Real Time PCR, obtaining low LOD, and analysing its applicability in several commercial foods (Ehlert, Hupfer, Demmel, Engel, & Busch, 2008; López-Calleja, Cruz, Gonz, García, & Martín, 2015b; Piknová & Kuchta, 2007). In this study, we additionally assayed the Real Time PCR system in boiled (30 or 60 min) and autoclaved samples in four different conditions of temperature/pressure (121 °C/1.18 atm or 138 °C/2.56 atm) and time (15 or 30 min), using Ana o 1 coding sequence as target and comparing classical TaqMan and LNA probes. Every treated flour was mixed in a matrix of spelt wheat (from 100000 to 10 mg of cashew per kg) and DNA from these binary mixtures was tested by PCR using 18S rRNA universal primers in order to confirm that quality and purity of the samples were suitable for amplification (data not shown).

Treatment based on boiling for 30 and even 60 min did not significantly affect the Real Time PCR performance. Curves from 100000 to 10 mg/kg of 30 min boiled samples showed good efficiency values (Fig. 3), and detection and quantification were possible even in the 10 mg/kg mixture. Similar results were achieved when cashew nuts were boiled for 1 h, although detection was approximately 1 cycle delayed when compared to untreated cashew nut curve. Ten ppm were detectable and quantifiable by Ana o 1 TaqMan probe system (Fig. 3A). LOD and LOQ values obtained from curves of several known amounts of boiled cashew nut flour in spelt wheat were very similar to those from unprocessed samples. In this regard, our group has reported abundant data over the influence of treatments (High Hydrostatic Pressure HHP, Boiling and Autoclave) on the amplification of allergen coding sequences of tree nuts by Real Time PCR, such as hazelnut, almond, walnut and pistachio (Iniesto et al., 2013; Linacero et al., 2016; Prieto et al., 2014; Sanchiz et al., 2017). HHP, based on the application of pressure without temperature, did not show reduction in almond detectability by Real Time PCR, although DNA fragmentation was observed by electrophoresis (Prieto et al., 2014). Similarly, boiling treatment did not affect the amplification of Pis v 1 target in pistachio by LNA probe-based Real Time PCR (Sanchiz et al., 2017), as well as Ana o 1 detection by using LNA or TaqMan probes-based Real Time PCR, described in this study, was not affected.

In contrast, Real Time PCR sensitivity was affected by increased temperature and time in autoclave treatment. Interestingly, Ana o 1 detection in cashew nuts autoclaved at 121 °C (1.18 atm) for 15 min also showed a good performance in both assays (Fig. 3), being possible to detect up to 10 mg/kg of cashew nuts and quantify up to 100 mg/kg (Fig. 3). There were significant statistical differences when calibration curve slope of untreated samples were compared with those of autoclaved samples 121 °C for 30 min, 138 °C for 15 min and 138 °C for 30min. Detection and quantification limits were established in 100 mg/kg for samples autoclaved at 121 °C for 30 min, the values of efficiency being acceptable when using TaqMan probe (105.89%), and in 1000 mg/kg when using LNA probe, with efficiency of 99.13% (Fig. 3). Surprisingly, this Real Time PCR method also detected cashew nuts (up to 1000 mg/kg) in significantly processed samples, such as autoclaving at 138 °C for 15 min (Fig. 3). Autoclave processing combines temperature with pressure, promoting large DNA degradation, and making DNA detection from an allergenic food or a genetically modified organism very difficult (Ballari & Martin, 2013). This fact implies that the Real Time PCR amplicon size should be short enough to achieve the best result of

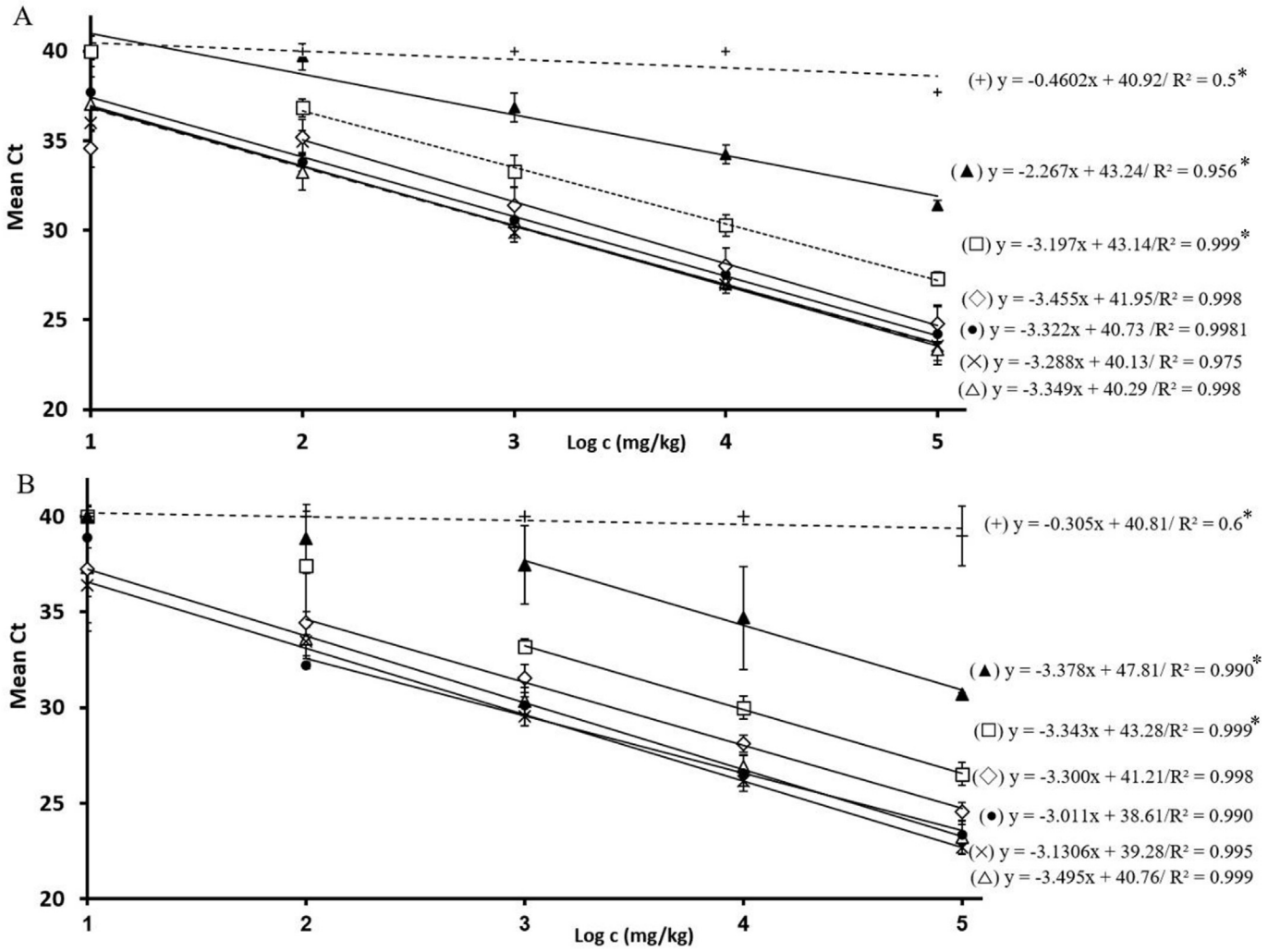

Fig. 3. Ana o 1 detection in treated cashew nut samples by Real Time PCR. Amplification of Ana o 1 target with A) TaqMan probe or B) LNA probe in binary mixtures of spelt wheat flour with known amounts (from 100000 mg/kg to 10 mg/kg) of untreated (Δ; E 98.87% for TaqMan and 93.27% for LNA) and treated cashew nut flour (X boiled 30′; E 101.43% for TaqMan and 93.95% for LNA; ● boiled 60′; E 99.98% for TaqMan and 108.65% for LNA; ◊Autoclave 121 °C 15′; E 94.72% for TaqMan and 100.91% for LNA; □Autoclave 121 °C 30′; E 106.89% for TaqMan and 99.13% for LNA; ▲ Autoclave 138 °C 15′; 97.71% for LNA; +Autoclave 138 °C 30′). Mean Ct values and standard deviations (n = 10 and 6 for untreated and treated samples respectively) for each spiked level are represented. Asterisk indicates significant differences when the calibration curves slopes from untreated and every treated sample in binary mixtures were compared ($p < 0.05$).

sensitivity if DNA integrity is strongly affected. In autoclave at 138 °C for 30 min treated samples, 10% of cashew nuts in spelt wheat matrix was detected by the Real Time PCR in 5 out of 6 replicates in Ana o 1 – TaqMan probe Real Time PCR.

Previously, performance of Real Time PCR assays has been described as strongly affected by thermal and pressure treatments (autoclave) in other tree nuts. In walnut, detection and quantification of Jug r 3 target was possible by Sybr-Green based Real Time PCR in autoclaved samples at 121 °C for 15 min, but detection was difficult in samples treated by autoclave at 138 °C (Linacero et al., 2016). In the same way, but with LNA probe-based Real Time PCR, detection and quantification of Pis v 1 target was drastically reduced in autoclaved samples at 121 °C for 15 min. This difference found in Real Time PCR performance between pistachio and cashew nuts using the same kind of probes (LNA) in processed samples might be due to the size of the amplicon designed in this work for cashew nuts, which was even smaller than Pis v 1 amplicon (Sanchiz et al., 2017).

Lopez-Calleja and collaborators performed a Real Time PCR system to detect ITS1 sequences from cashew nuts and macadamia nuts using TaqMan probes, with promising results regarding specificity and high sensitivity. The authors obtained the same results with roasted nuts at 160 °C for 13 min, a typical treatment in ready-to-eat cashew nuts (López-Calleja et al, 2015a). In our experience, this Real Time PCR system for cashew nuts is the first one that is able to detect and quantify cashew nut traces after being subjected to heat and pressure at 121 °C for 30 min, maintaining linearity up to 100 ppm, and an adequate efficiency. Ana o 1 target was detected in mixtures with 1000 ppm of cashew nuts treated by autoclave at 138 °C for 15 min in all replicates when TaqMan probe was used, and in 66% of the replicates with the LNA probe-based system. Finally, in harsher autoclave conditions than before (138 °C for 30 min), these Real Time PCR systems did not detect the target in any replicate when the quantity of treated cashew nuts in the mixture was less than 10% w/w.

Although both assays with different probes showed the same limit of detection and quantification, established in 10 mg/kg of cashew nuts, data suggested that TaqMan probe allowed to detect low quantities of untreated cashew nuts better than LNA (5 ppm). A very similar performance was obtained with both probes when

treated cashew nuts were assayed, although TaqMan probe can also detect treated cashew nuts better than LNA-based system when the quantity of the nut is low.

3.4. Detection of traces of cashew nuts in commercial food products

The applicability of Ana o 1 as a target to detect cashew nuts by Real Time PCR, comparing both hydrolysis probes, has been determined in several commercial food products, including chocolate, meat, cookies, cereal bars, sauce, etc (Table 3). Two of them declared cashew nut presence in the label, eight declared presence of other nuts or may contain traces from nuts, and three did not declare presence of any nut. All food samples were first amplified with 18S rRNA primers as false negative control, obtaining maximum Ct values of 25 in those foods with strong DNA fragmentation after harsh processing conditions. ELISA was also performed with such products in order to compare both techniques (Table 3). The correspondence between Real Time PCR assays and ELISA to detect cashew nut traces was 9 out of 13 food products. Commercial ELISA kit detected three false positives in food products with 10% of hazelnut and 5% of pistachio (this cross-reactivity with pistachio is actually indicated in the AgraQuant ELISA instructions). Contrarily, same products analysed by Real Time PCR provided Ct values above 40 (not detected) when both kind of hydrolysis probes were assayed, demonstrating better specificity for cashew nut detection than commercial ELISA kit (Table 3).

In contrast, mean Ct values around 38 were obtained with both probes for one of the foods, named *"cookies with chocolate"*, which did not declare nut presence in the label. According to the kit instructions, limit of detection and quantification of AgraQuant ELISA for cashew nut detection was 0.2 mg/kg and 2 mg/kg, respectively; however, ELISA did not detected cashew nuts in this food, "cookies with chocolate", probably due to the excessive processing applied on cashew nut proteins. Substitution of the obtained mean Ct values in the calibration curves for LNA and TaqMan-based real time PCR systems provided an estimation of 3.74 and 5.25 mg/kg of cashew (Table 3). A standard curve should be performed for any kind of processed matrix in order to develop a real quantitative assay for cashew nut content, but this is not feasible from a practical point of view (López-Calleja et al, 2015a). As explained before, both assays were able to detect and quantify 5 ppm of cashew nuts in binary mixtures (Fig. 2) with Ct values of 38, so adventitious contamination with cashew nuts might have occurred in the factory where this food was produced.It should be noticed that Real Time PCR assay using TaqMan probe provided positive amplification with 25 ng of almond DNA; however, specific applicability of this method has been confirmed when food with almond as ingredient was assayed (Table 3).

The substitution of the obtained Ct in the standard curve (binary mixtures) allows an estimation of the quantity of cashew nuts in mg/kg in analysed food products. For the two food samples with declared cashew nut presence in the label (pesto and cookies with 5% and 6% of cashew nuts, respectively), the estimated amount was 79,432 - 103,000 mg/kg for pesto (around 8–10%) and about 50,000 mg/kg (5%) for cookies. The differences between the estimated percentage of cashew nuts and the percentages in labels might be due to a possible mistake in labelling or, likely, to the presence of one or more compounds in the food matrix, which interfere with the amplification reaction (Opel, Chung, & McCord, 2010). These two cashew based foods were also measured by the commercial ELISA kit, providing an estimated cashew quantity of around 50 mg/kg and 60 mg/kg for pesto and cookies respectively. These estimated values are clearly below the declared amount in such foods. Therefore, Real Time PCR gave rise to more sensitive results compared to commercial ELISA. Comparing both hydrolysis targets, no important differences have been obtained about the applicability of this Real Time PCR system to detect Ana o 1 target and, therefore, both are equally valid to detect cashew nuts in foodstuff.

4. Conclusions

In this study, two Real Time PCR systems have been performed in order to detect cashew nut Ana o 1 allergen-coding sequence in processed food, with an established limit of detection and quantification of 10 ppm. The main goal of this work was cashew nut detection in highly processed samples, by boiling and autoclave. Boiling did not affect the DNA integrity and had no effect on cashew nut detectability with both probes. For the first time, a Real Time PCR assay is able to detect and even quantify up to 0.1% of extremely processed cashew nuts (autoclave at 138 °C/2.56 kPa for 15 min) in complex mixtures. Other novelty of this study has been the comparison of efficiency, sensitivity, specificity and applicability between two hydrolysis probes for the detection of the same target by Real Time PCR method. According to our general results, both LNA and classical TaqMan probes are suitable for reliable and specific detection of cashew nuts in complex mixtures, processed samples and commercial food products. Real Time PCR, compared to commercial ELISA test, gave rise to more specific and sensitive results.

Table 3
Detection of cashew by real time PCR using LNA and TaqMan probes for Ana o 1 allergen coding sequence and ELISA in commercial food products.

Sample	Label declaration	Ana o 1 LNA[a]	Cashew (mg/kg)[b]	Ana o 1 TaqMan	Cashew (mg/kg)[b]	18S rRNA	ELISA[c] AgraQuant
Cereal bar I	Almond	Not detected	–	Not detected	–	17.57 ± 0.46	- (1.6)
Cereal bar II	Might contain nuts	Not detected	–	Not detected	–	22.35 ± 2.25	- (1.6)
Cereal bar III	Hazelnut	Not detected	–	Not detected	–	17.78 ± 0.38	3.1
Chocolate with hazelnut	Hazelnut	Not detected	–	Not detected	–	18.42 ± 1.67	10.9
Black chocolate	Might contain nuts	Not detected	–	Not detected	–	18.73 ± 0.03	- (<LOD)
Cookies cereal and chocolate	Might contain hazelnut	Not detected	–	Not detected	–	20.17 ± 1.05	-(<LOD)
Cookies with fiber	Nuts not declared	Not detected	–	Not detected	–	24.92 ± 0.63	- (<LOD)
Chocolate bar	Nuts not declared	Not detected	–	Not detected	–	20.71 ± 1.50	- (<LOD)
Cookies with chocolate	Nuts not declared	38.02 ± 0.89	3.74	38.00 ± 0.88	5.25	20.62 ± 0.25	- (<LOD)
Chocolate with pistachio	Pistachio, almond, hazelnut	Not detected	–	Not detected	–	12.06 ± 0.20	27.9
Vegetal burguer	Might contain nuts	Not detected	–	Not detected	–	15.32 ± 0.26	- (<LOD)
Pesto sauce	5% cashew	23.09 ± 0.30	103,000	23.80 ± 0.57	79,432	14.73 ± 0.10	54.2
Cookies with cashew	6% cashew	24.11 ± 0.10	51,286	24.70 ± 0.13	44,668	15.22 ± 0.13	61.4

[a] Average Cycle threshold (Ct) ± standard error.
[b] Estimated cashew quantity by substitution in standard curve from binary mixtures with mean Ct value.
[c] ELISA results expressed as mg/kg cashew. Values < 2 mg/kg are negative according to the manufacturer instruction.

5. Declarations of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.foodcont.2018.02.021.

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Appendix 6. Supplementary Material

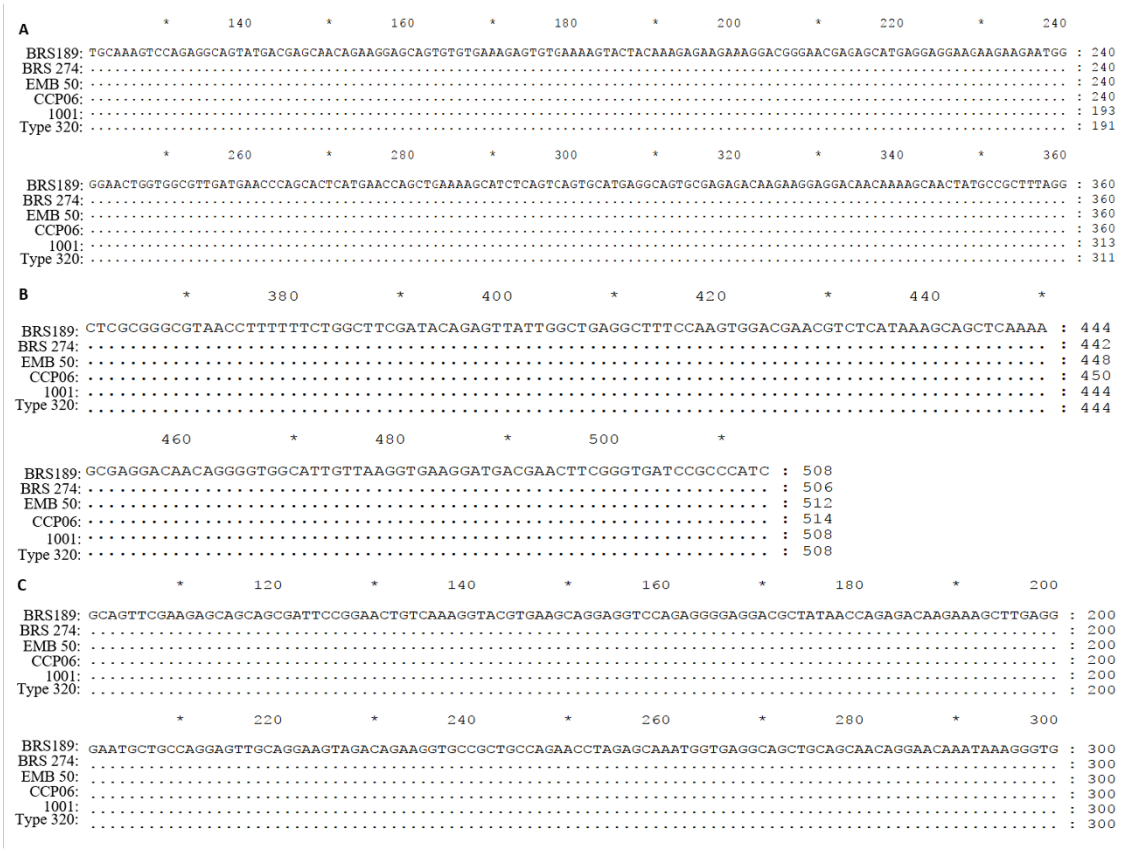

Figure 1. Supplementary material. Partial alignment of Ana o 1 (A), Ana o 2 (B) and Ana o 3 (C) coding gene DNA sequences from six cashew nut varieties.

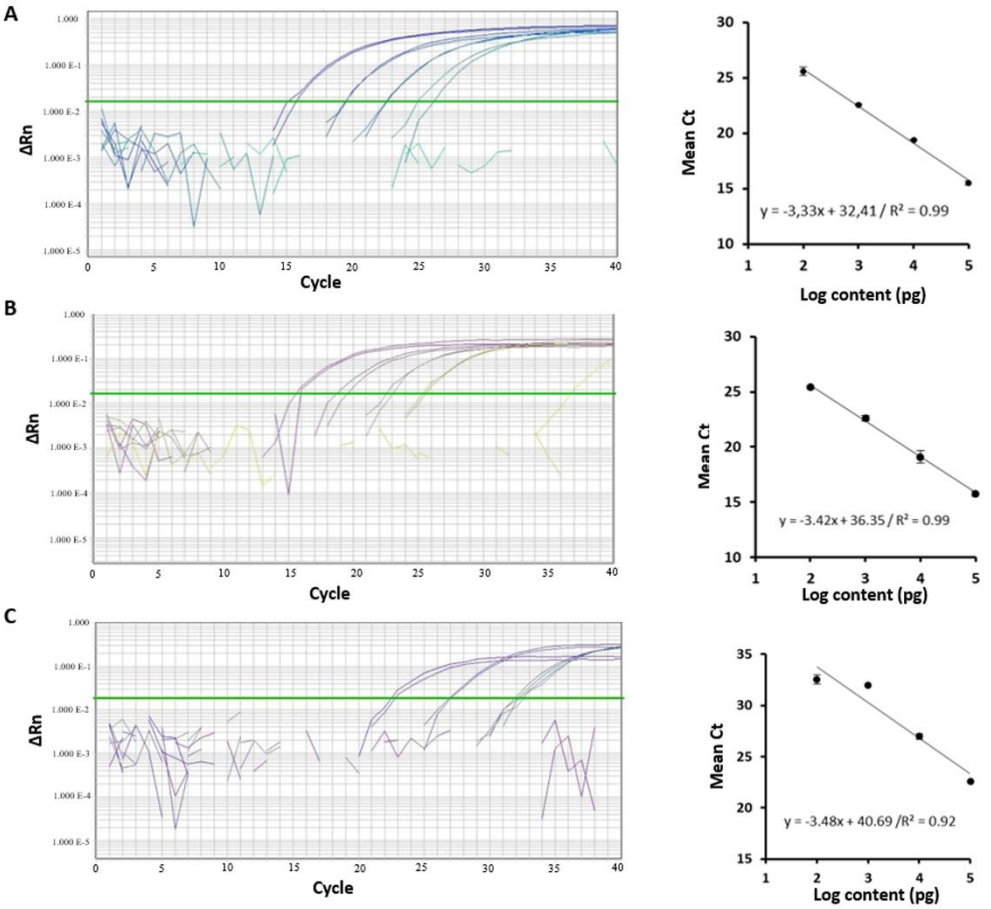

Figure 2. Supplementary material. Amplification plots and calibration curves from cashew nut DNA, using Ana o 1 (A), Ana o 2 (B) and Ana o 3 (C) primers in LNA probe-based real time PCR.

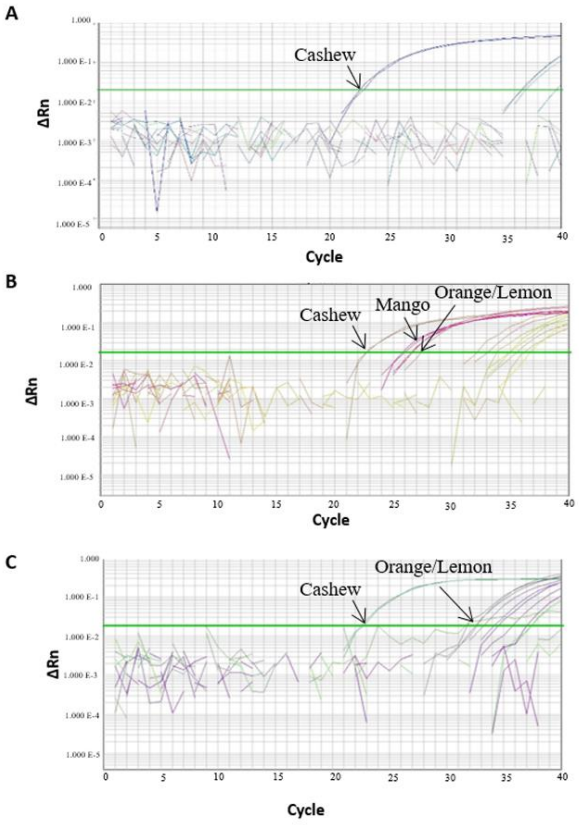

Figure 3. Supplementary material. Amplification plots from cashew and other plant species DNA using Ana o 1 (A), Ana o 2 (B) and Ana o 3 (C) primers in LNA probe-based real time PCR.

DISCUSIÓN INTEGRADORA

EFECTO DEL PROCESADO TÉRMICO, DE PRESIÓN Y ENZIMÁTICO SOBRE LA INMUNOREACTIVIDAD DE FRUTOS SECOS

Cocción y autoclave

A nivel de reactividad frente a IgE, la influencia de los tratamientos térmicos y de presión sobre anacardo y pistacho se ha evaluado mediante pruebas *in vitro* empleando un grupo de sueros de pacientes alérgicos a estos frutos secos, que, aunque limitado en número, ha sido bien caracterizado. Una parte de los ensayos se ha llevado a cabo empleando una mezcla (*pool)* de un total de 6 sueros españoles, reclutados por el Hospital Universitario 12 de Octubre de Madrid (resultados descritos en el Artículo 1). Por otro lado, se ha realizado una evaluación mediante ensayos *in vitro* empleando, individualmente, 7 sueros de origen norteamericano procedentes del Tulane Health Science Center (Nueva Orleans) (resultados descritos en el Artículo 2). Se han observado ciertas diferencias entre los dos trabajos relativos al nivel de inmunoreactividad y el patrón de bandas reactivas frente a IgE. Esto puede ser debido fundamentalmente a que la evaluación de la capacidad inmunoreactiva empleando sueros humanos puede dar lugar a resultados diferentes entre individuos, poblaciones o países de procedencia de los mismos. Se han establecido diferencias significativas de sensibilización a cacahuete entre EEUU, España y Suecia, en cuanto a la reactividad frente a las distintas proteínas alergénicas Ara h (Vereda *et al*, 2011). El perfil de sensibilización de pacientes alérgicos a melocotón del norte de España es diferente entre distintos individuos y se han identificado diferencias en los patrones inmunológicos y clínicos de alergia a ciertos frutos secos entre provincias de España (Gamboa *et al*, 2007; Haroun-Díaz *et al*, 2017).

Los tratamientos térmicos pueden provocar la agregación proteica formando oligómeros y comprometiendo la solubilidad de las proteínas, incluidos los alérgenos, lo que alteraría las propiedades antigénicas de un extracto proteico (Maleki, 2004; Mills *et al*, 2009; Masthoff *et al*, 2013). Para tener en consideración este problema se han realizado experimentos no solo con extractos proteicos, sino también con harinas y pasta de harina desengrasada, con el fin de obtener las conclusiones más precisas y fiables.

Los resultados descritos permiten concluir que la cocción, durante 30 y 60 minutos, no conlleva modificaciones sustanciales en el perfil electroforético respecto al control y produce un leve descenso en la capacidad de unir IgE de pacientes alérgicos si se compara con la muestra sin tratar. Tanto en los ensayos realizados con una mezcla de sueros españoles como con sueros individuales norteamericanos, se detectaron múltiples bandas inmunoreactivas de anacardo y

pistacho tratados con cocción, de forma similar al control, incluso tras 60 minutos. Los resultados obtenidos indican que los alérgenos de estos frutos secos analizados muestran poca susceptibilidad a la cocción. Las proteínas alergénicas de anacardo, de almendra y de nuez demostraron una elevada estabilidad antigénica tras someter los frutos a radiación gamma seguido de una amplia variedad de tratamientos térmicos, incluyendo cocción 5 y 10 minutos (Su *et al*, 2004).

Noorbakhsh y colaboradores (2010) describieron que el cocinado al vapor de frutos de pistacho produce una menor capacidad de unir IgE, en relación a la que se consigue aplicando tostado seco. La aplicación de calor en condiciones húmedas ha demostrado ser también más eficaz a la hora de afectar a la inmunoreactividad de los alérgenos de cacahuete comparado con calor seco (tostado), probablemente debido a la lixiviación de las proteínas al agua de tratamiento (Mondoulet *et al*, 2005; Cabanillas *et al*, 2012a). El remojo de frutos de cacahuete previo a la aplicación de autoclave ha resultado esencial para provocar una disminución importante de las propiedades inmunoreactivas de este fruto (Bavaro *et al,* 2018). Ciertas leguminosas como lenteja y garbanzo, en las que la cocción es un tratamiento habitual para su preparación, han demostrado estabilidad antigénica tras el procesado térmico con respecto al control (Cuadrado *et al,* 2009) o incluso un incremento de reactividad frente IgE (Sánchez-Monge *et al*, 2000).

La literatura previa ha demostrado que la combinación de temperatura y presión, en forma de autoclave, produce reducciones sustanciales de la capacidad inmunoreactiva de nuez, cacahuete y otras leguminosas como lupino o lenteja, evaluada mediante diferentes técnicas (Álvarez-Álvarez *et al*, 2005; Cuadrado *et al*, 2009; Cabanillas *et al*, 2012a; Cabanillas *et al*, 2014; Cabanillas *et al,* 2015). Por ello, se ha profundizado en la aplicación de este tratamiento térmico con presión sobre pistacho y anacardo, como posible método efectivo para reducir su capacidad alergénica. Se han empleado distintas condiciones de presión (1.18 y 2.56 atm/120 y 256 kPa), temperatura (121 y 138^{o}C) y tiempo (15 y 30 minutos), en condiciones húmedas, ya que los frutos estaban sumergidos en agua destilada durante el tratamiento.

El patrón electroforético está gradualmente más afectado a mayor temperatura y tiempo, observándose una disminución de bandas proteicas por encima de 35 kDa en ambos frutos secos. Las condiciones de autoclave a 121^{o}C/1.18 atm o 120 kPa, 30 min reduce la capacidad de los alérgenos de pistacho y anacardo de unir IgE, en especial en el grupo de sueros españoles, sin embargo, se detectan bandas inmunoreactivas y cierta capacidad de competir por IgE frente al extracto control (especialmente en pistacho). Con el grupo de sueros norteamericanos no se experimenta una reducción relevante de unión a IgE, evaluado por

Western blot. Su et al (2004) concluyeron que el someter a varios frutos secos a autoclave a 121^{o}C durante 20 min, no conllevaba cambios importantes en la antigenicidad de los mismos, lo que corroboraron posteriormente Venkatachalam y colaboradores (2008) estudiando los alérgenos de anacardo de manera individual.

Las muestras de pistacho y anacardo tratadas por autoclave a 138^{o}C/2.56 atm muestran una mayor acumulación de péptidos en la zona de bajo peso molecular y una drástica modificación del perfil electroforético de pistacho y anacardo. De manera general, los ensayos realizados con sueros de pacientes españoles y americanos muestran una reducción considerable de la capacidad de inmunoreaccionar de ambos frutos tratados con autoclave a 138^{o}C, siendo más patente en las condiciones extendidas durante 30 minutos. Sin embargo, mediante ELISA inhibición se ha detectado que el extracto autoclavado retiene cierta capacidad de unir anticuerpos IgE del pool de sueros español, compitiendo con el extracto de muestras sin tratar. Un pequeño porcentaje de sueros procedentes de Norteamérica reconocieron bandas en estas muestras autoclavadas de pistacho y anacardo, siendo las bandas de 32 y 13 kDa en anacardo (posiciones esperada de Ana o 2 (subunidad ácida) y Ana o 3 respectivamente) y fundamentalmente las de 35 kDa y 25 kDa en pistacho (Pis v 2) las más resistentes. La desnaturalización de las proteínas alergénicas como consecuencia del procesado térmico y de presión, puede enmascarar o destruir irreversiblemente epítopos con capacidad de unir IgE, especialmente los conformacionales (Rahaman *et al*, 2016).

Los basófilos, junto a los mastocitos, son células efectoras clave en alergia y otras enfermedades inflamatorias. Los modelos celulares basados en líneas de basófilos de rata como RBL (Rat Basophil Leukemia) expresan el receptor de alta afinidad humano FcεRI y han sido descritos como sistemas útiles, sensibles e informativos a la hora de estudiar alérgenos, interacciones IgE-alérgeno y la capacidad potencial de un alérgeno para desencadenar una respuesta alérgica (Dibbern *et al*, 2003; Sun *et al*, 2015). En estos modelos se mide la activación y liberación de mediadores de la respuesta alérgica (ensayo MRA, *mediator release assay).* En la figura 8 se esquematiza los pasos del ensayo MRA realizado en este trabajo (*Artículo 1*).

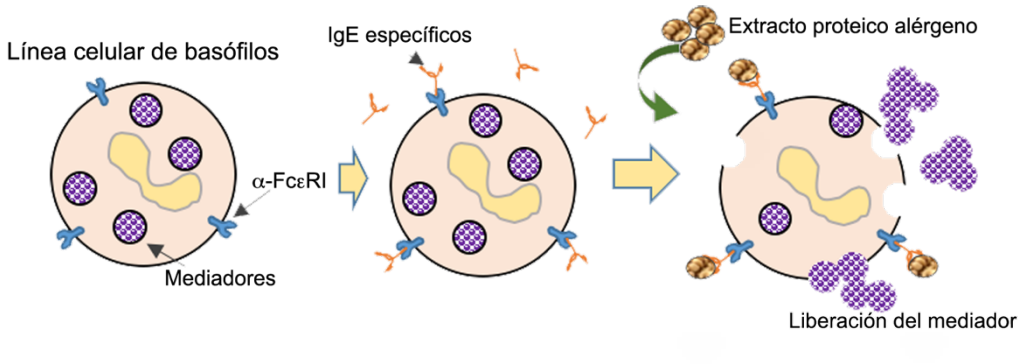

Figura 8. Esquema del ensayo de liberación de mediadores (MRA) en la línea celular humanizada de basófilos de rata RBL-48.

Los resultados descritos muestran que la estimulación de basófilos de la línea celular RBL con el extracto proteico de las muestras cocidas 60 min desencadenó una considerable menor liberación de β-hexosaminidasa respecto al control (60%). Las pruebas cutáneas con extractos proteicos de muestras cocidas realizadas en el grupo de pacientes españoles revelaron también un menor tamaño de la pápula con respecto al control, pero el resultado fue positivo en la mayoría de los pacientes evaluados (>3 mm). Este tratamiento por tanto reduce, pero no anula, la capacidad de inducir sintomatología alérgica en los pacientes (*Figura 1 y Figura 4 del Artículo 1)*.

Ningún paciente reaccionó positivamente en las pruebas cutáneas frente a extracto de anacardo y pistacho tratados con autoclave a 121^{o}C 30 min, 138^{o}C 15 y 30 min. Se observó también una reducción sustancial en la liberación de mediadores por parte de la línea RBL al aplicar los tratamientos de autoclave en anacardo y pistacho, comparado con la muestra control. Se observa cierta capacidad estimuladora y liberadora de β-hexosaminidasa en anacardo tratado con autoclave, ligeramente superior en las condiciones 138^{o}C 30 min que el resto de tratamientos. Los ensayos de inmunodetección *in vitro* (WB, ELISA) empleados para evaluar los cambios en la capacidad de los alérgenos alimentarios para unir IgE, no siempre correlaciona con una alteración en la capacidad de los mismos para unir IgE en un sistema más complejo (SPT y MRA) y desencadenar una respuesta mediada por IgE (Vissers *et al*, 2011; Shi *et al*, 2013; Sun *et al*, 2015).

Los epítopos de alérgenos alimentarios más estudiados han sido los lineales, principalmente debido a la mayor complejidad de estudio que requiere analizar los conformacionales (Matsuo *et al*, 2015). Ambos tipos de epítopos, lineales y conformacionales, son importantes a nivel inmunoreactivo en frutos secos, tal y como se ha demostrado en nuez o almendra (Robotham *et al*, 2002; Willison *et al*, 2013). En anacardo se ha caracterizado un

epítopo conformacional en el alérgeno Ana o 2, con una relevante implicación en la unión a IgE, evaluada *in vitro* (Robotham *et al*, 2010). De forma general, el procesado de los alimentos puede inducir la modificación estructural de los epítopos conformacionales, por lo que algunos autores han destacado el mayor papel inmunoreactivo de los lineales en alergia alimentaria, más resistentes al procesado (Vila *et al*, 2001; Dhakal *et al*, 2014).

Se ha realizado un análisis preliminar de los péptidos que han resistido al tratamiento extremo de autoclave aplicado (Tablas A1 y A2 del anexo). Mediante un análisis de espectrometría de masas, realizado en la zona de migración electroforética entre 10 y 15 kDa de pistacho y anacardo tratados (Figura A1 del anexo), se han identificado péptidos de alérgenos principales de pistacho y anacardo (Ana o 2 y Ana o 3, Pis v 1, 2, 3 y 5). Estos péptidos muestran resistencia al tratamiento de calor y presión por autoclave a 138^{o}C/2.56 atm durante 30 minutos. En anacardo se han descrito epítopos de unión a IgE (Wang *et al*, 2002, 2003; Robotham *et al*, 2005), lo que permite comprobar si los péptidos resistentes al tratamiento corresponden total o parcialmente a esos epítopos descritos (Tabla A1). La secuencia de algunos de los péptidos de anacardo que han resistido al tratamiento solapan, total o parcialmente, con la secuencia aminoacídica de esos epítopos lineales inmunoreactivos descritos. Su presencia podría ser capaz de degranular los basófilos al unirse a los anticuerpos IgE anclados en su membrana, aunque de forma visiblemente menos eficiente que el control sin tratar (Robotham *et al*, 2002; Albrecht *et al*, 2009). En el caso de pistacho, los epítopos lineales de relevancia inmunoreactiva no están descritos, por lo que esta comparación no es posible. Se han comparado los péptidos resultantes de pistacho con las secuencias de epítopos de anacardo, dada la homología existente entre las secuencias aminoacídicas de sus alérgenos (Figura A2 del anexo). Algunos de estos péptidos se localizan en las mismas regiones que los epítopos de anacardo, compartiendo parte de su secuencia de aminoácidos.

Tratamiento térmico e hidrólisis enzimática

El uso de proteasas en industria alimentaria abre un amplio abanico de aplicaciones, entre las que se encuentra reducir el potencial alergénico de ciertos compuestos (Tavano, 2013). Las enzimas del aparato digestivo (principalmente pepsina, tripsina, α-quimotripsina) se emplean para evaluar *in vitro* la resistencia a la digestión de alérgenos de frutos secos y la influencia de la digestión sobre su alergenicidad (Eiwegger *et al*, 2006). En este estudio, se ha evaluado la susceptibilidad de las proteínas de anacardo y pistacho (control, cocción 60' y autoclave 121^{o}C 30 min) a la digestión con las endopeptidasas digestivas pepsina (estómago) y tripsina (intestino) mediante electroforesis, analizando el posible efecto del procesado térmico

con y sin presión sobre el nivel de hidrólisis. En la búsqueda de estrategias potencialmente eficientes para la obtención de anacardo y pistacho hipoalergénicos, se ha planteado la aplicación combinada de los tratamientos térmicos de cocción y autoclave con hidrólisis con proteasas de grado alimentario (exo y endoproteasas) y se ha analizado el efecto de estos tratamientos sobre la inmunoreactividad, mediante inmunodetección *in vitro*. Para ello, se ha utilizado el grupo norteamericano de pacientes alérgicos a anacardo y pistacho (Artículo 2).

Las condiciones seleccionadas para las hidrólisis enzimáticas realizadas en este trabajo se resumen en la tabla 3.

Tabla 3. Condiciones de hidrólisis enzimática aplicados sobre pistacho y anacardo en este trabajo.

Enzima	Muestra	$[proteína]_{final}$	$[enzima]_{final}$	Tª/pH	Tiempo
Pepsina	P/A - extracto BSB	0.4 mg ml^{-1}	0.01 μM -SGF	37ºC / 1.2	Hasta 1.5 h
Tripsina	P/A – extracto BSB	0.4 mg ml^{-1}	0.042 μM - SIF	37ºC / 8.3	Hasta 16 h
Proteasas E1-E7	P/A – extracto BSB	2 mg ml^{-1}	1 mg ml^{-1}- PBS	55ºC / 7.4	Hasta 19 h
E5-sonicación	P – pasta de harina	500 mg ml^{-1} (*)	11 μg ml^{-1}- DW	55ºC / 7.4	1h
E7-sonicación	A – pasta de harina	500 mg ml^{-1} (*)	11 μg ml^{-1}-DW	55ºC / 7.4	2 h

(*)Pasta de harina completa en agua destilada (DW). SGF, pepsina (P7012, Sigma-Aldrich. Fluido gástrico simulado 84 mM HCl, 35 mM NaCl), SIF, tripsina (T1426, Sigma-Aldrich. Fluido intestinal simulado 50 mM Tris Base, 1 mM EDTA).

La resistencia que tienen los alérgenos alimentarios a la digestión mediada por enzimas es una de sus principales características (Bannon *et al*, 2002). En este trabajo, se muestra que la digestión individual con pepsina induce una acumulación de péptidos y proteínas por debajo de 25 kDa, mientras que la mayoría de proteínas de mayor peso molecular desaparecen y no son visibles en los primeros 15 minutos de hidrólisis (Figura A3 del anexo). En ambos frutos secos, tras 90 minutos de hidrólisis con pepsina se visualiza un incremento de proteínas de menos de 15 kDa, en bandas difusas, correspondiente a una acumulación de péptidos y proteínas de bajo peso molecular. Los resultados de SDS-PAGE reflejan que la tripsina, a las condiciones ensayadas, ejerce menor efecto en el patrón electroforético de anacardo y pistacho que la pepsina. Los resultados concuerdan con los obtenidos en anacardo por Mattison *et al* (2014). Estos autores demuestran que la concentración de enzima es un factor clave para que la proteólisis sea efectiva y concluyen que Ana o 3, la albúmina 2S de anacardo, mantiene su integridad y capacidad de unión a IgE bajo determinadas condiciones de digestión secuencial con pepsina y tripsina. En nuestro estudio, la digestión con pepsina y tripsina de muestras tratadas con cocción 60 min y autoclave a 121ºC 30 min no parece aumentar la sensibilidad de anacardo frente a la hidrólisis con las peptidasas (Figura A3). Este efecto es similar en pistacho con pepsina, sin embargo, la hidrólisis con tripsina resultó ligeramente más eficiente en muestras de pistacho térmicamente tratadas (Figura A3-B del anexo). El efecto de la hidrólisis *in vitro* con enzimas digestivas sobre la inmunoreactividad frente a IgE se ha realizado previamente

en otros frutos secos. La digestión con pepsina de las globulinas Ara h 1 y Ara h 3 es mucho más efectiva que sobre las albúminas Ara h 2 y Ara h 6 de cacahuete, incluso a altas concentraciones de enzima, lo que se explica por la estabilidad propia de las albúminas (Moreno & Clemente, 2008; Koppelman *et al*, 2010). Venkatachalam *et al* (2006) han comprobado que ciertas proteínas de nuez pecana, resistentes a la simulación SGF, se mantienen además antigénicas. La nuez tratada con autoclave a 121^{o}C 30 min, por su parte, resultó más susceptible al tratamiento combinado de tripsina y α-quimotripsina que el correspondiente control, de acuerdo a Cabanillas y colaboradores (2014). Dado que la influencia de las enzimas ensayadas en este trabajo no ejerce una gran destrucción proteolítica en anacardo y pistacho, sería conveniente realizar hidrólisis secuencial de pepsina + tripsina/α-quimotripsina, además de evaluar el efecto sobre la inmunoreactividad IgE sobre anacardo y pistacho, tal y como se ha realizado en estudios previos (Cabanillas *et al*, 2010; 2012b).

Se ha planteado el uso de enzimas de grado alimentario con las que se ha desarrollado un nuevo protocolo de hidrólisis seleccionando las más efectivas para anacardo o pistacho, de entre una batería de proteasas (E1-E7, Amano Enzyme Europe) y distintos tiempos de digestión. Los experimentos se realizaron en extractos proteicos y en pastas de harinas desengrasadas de ambos frutos secos; en este último caso se requirió la aplicación de ultrasonicación para favorecer el proceso de digestión y reducir el tiempo de tratamiento (*Artículo 2*). La selección y optimización de estos parámetros resulta esencial para encontrar las condiciones más propicias que reduzcan la capacidad inmunoreactiva de las fuentes alergénicas (Hur *et al*, 2011). La digestión con proteasas y sonicación de harinas desengrasadas de anacardo (tratado con proteasa "Amano 3DS" durante 2 horas de sonicación) y pistacho (con endoproteasa tipo alcalasa durante 1 hora de sonicación), produjeron hidrólisis peptídica eficiente, particularmente en proteínas por encima de 25 kDa.

A nivel inmunoreactivo, se muestra que la hidrólisis con proteasas en las harinas control disminuyó la capacidad de los alérgenos de unir IgE, siendo más susceptibles las proteínas de pistacho. Como resultado de la digestión enzimática pueden destruirse epítopos lineales, si bien la resistencia de los mismos va a depender en gran parte del grado de hidrólisis y de la enzima empleada (Rahaman *et al*, 2016). La influencia de la hidrólisis enzimática sobre la alergenicidad de alimentos ha sido objeto de numerosos estudios. La digestión secuencial con endo y exoproteasas (Alcalasa y Flavorzima, respectivamente) sobre extracto de lenteja trajo consigo una importante destrucción de epítopos capaces de unir IgE, aunque no completa (Cabanillas *et al*, 2010). El cacahuete tostado hidrolizado presentó menor habilidad para unir IgE de sueros de

pacientes alérgicos que el control; sin embargo, mantenía sus propiedades de activación de basófilos prácticamente intactas (Shi *et al*, 2013).

Al aplicar proteasas de grado alimentario sobre muestras tratadas previamente con calor y presión, se ha observado una reducción efectiva de la reactividad de anacardo y pistacho, probablemente debido a que el tratamiento por calor induce la desnaturalización de las proteínas, exponiendo sitios de corte de las proteasas y promoviendo la destrucción de epítopos de unión a IgE (Clemente, 2000; Rahaman *et al*, 2016). El pistacho y anacardo sometidos a cocción 60 min y proteasa presentaron capacidad para unir IgE muy limitada, siendo aún más reducida con autoclave 138^{o}C 30 min, en la que sólo uno de los pacientes alérgicos a pistacho o anacardo inmunoreaccionó con proteínas alergénicas. La combinación de tratamiento térmico y enzimático permite reducir el potencial alergénico del huevo, sin afectar a propiedades organolépticas (Hildebrandt *et al*, 2008). La simulación del fluido gástrico y duodenal sobre extractos de castaña (*C. crenata)* junto con tratamiento térmico de cocción durante 5 minutos redujo significativamente la capacidad inmunoreactiva de este fruto (Lee *et al*, 2005).

Se ha podido concluir que algunos péptidos resisten al tratamiento de hidrólisis, incluso en combinación con tratamiento térmico. Según el análisis de espectrometría de masas realizado, Pis v 2 (legumina 11S) y en menor medida Pis v 1 (albúmina 2S) son los dos alérgenos más resistentes al tratamiento combinado en pistacho. En anacardo, se detectaron péptidos resistentes al tratamiento combinado, correspondientes a Ana o 2 (legumina 11S), en la zona electroforética e inmunoreactiva de 20 kDa. En el tratamiento a las condiciones más extremas, AU 138^{o}C 30 min y proteasa, se detectaron péptidos de de Ana o 2 como de Ana o 3 (albúmina 2S) en la zona de menor peso molecular. Estos alérgenos sufren degradación, dando lugar a fragmentos más pequeños, que resisten el procesado y son detectables en la zona de 6-10 kDa, lo que remarca de nuevo la alta estabilidad térmica y a la hidrólisis enzimática de estas proteínas. Algún péptido detectado e identificado al analizar pistacho y anacardo únicamente autoclavados (138^{o}C 30 min) sigue siendo identificable en las muestras autoclavadas y posteriormente digeridas enzimáticamente, demostrando una alta resistencia al procesado proteolítico. Estos péptidos coincidentes en muestras tratadas con autoclave 138^{o}C 30 min con y sin tratamiento con proteasas fueron (R)FEWISFK(T), (R)LTTLNSLNLPILK(W), (R)TSVLGGMPEEVLANAFQISR(E) y (R)ADIYTPEVGR(L) de Ana o 2, (K)LQELYETASELPR(M) de Pis v 1 y (R)FLQLSAK(K) de Pis v 2 (Tablas A1 y A2). Algunos de éstos han sido identificados por Mattison *et al* (2014) como resistentes a la hidrólisis con pepsina y tripsina, determinando además que Ana o 3 era el alérgeno más resistente a la digestión enzimática. Nuestros datos corroboran estos resultados y demuestran

que Ana o 2 es también una proteína muy resistente al tratamiento, en este caso, combinando temperatura, presión y digestión enzimática con proteasas.

Influencia de cocción y autoclave sobre otras propiedades de frutos secos

Para el posible uso de harinas de frutos secos con propiedades inmunoreactivas reducidas en aplicaciones alimentarias es recomendable analizar los cambios producidos en otras propiedades, de tipo nutricional y/o funcional, consecuencia del tratamiento. Es conocido que el procesado alimentario afecta a ciertos componentes de los alimentos, de interés desde un punto de vista nutricional, como los compuestos fenólicos (Pedrosa *et al*, 2015; López-Martínez *et al*, 2017), sustancias asociadas a propiedades beneficiosas para la salud, con funciones antimicrobianas, anticancerígenas y antiinflamatorias y antioxidantes (Yao *et al,* 2004; Cardador-Martínez *et al,* 2014). En este trabajo se ha analizado la cantidad de compuestos fenólicos (fenoles totales, antocianinas, flavonoles y esteres tartáricos) de tres frutos secos en los que se ha probado una disminución de las propiedades antigénicas tras aplicar tratamiento térmico y de presión: anacardo, pistacho y castaña, de la que previamente se han obtenido resultados semejantes mediante pruebas inmunológicas *in vitro* e *in vivo* (Gimenez-Licitra *et al*, 2018; De las Cuevas *et al*, 2018). Se ha determinado también la actividad antioxidante mediante dos ensayos, ORAC (*Oxygen Radical Absorbation Capacity)* y DMPD (*Trolox Equivalent Antioxidant Capacity).*

Los tratamientos térmicos y de presión aplicados han influido sobre el contenido de los compuestos fenólicos analizados. La cocción reduce el contenido de fenoles totales, flavonoles y ésteres tartáricos entre un 30-45%, en pistacho y anacardo comparado con el control. Este efecto no se observa en castaña. El tratamiento térmico puede favorecer la salida de determinados componentes al agua de cocción. Cabe esperar que las propiedades físico-químicas del fruto, el espesor de la cubierta, el tamaño o la dureza de la semilla confieran mayor o menor susceptibilidad al procesado aplicado (Siah *et al*, 2014). Algunas legumbres cocidas también experimentan una pérdida de compuestos fenólicos derivada del procesado, observándose lo mismo en su actividad antioxidante (Xu & Chang, 2008). Al aplicar autoclave, el contenido de compuestos fenólicos de los tres frutos secos analizados se incrementa con la temperatura/presión y tiempo, alcanzando valores superiores al control en el contenido de fenoles totales de castaña autoclavada a 138^{o}C 30 minutos. Chandrasekara y Shahidi (2011) mostraron un incremento en la cantidad de fenoles totales (TPC) en frutos de anacardo tostado a alta (133^{o}C 30 min), con respecto al no tratado. En pistacho se han obtenido resultados

similares tras determinadas condiciones de tostado, incrementándose el contenido total de polifenoles (Rodriguez-Bencomo *et al*, 2015).

Las proteínas pueden formar complejos con compuestos fenólicos, induciendo cambios en determinadas propiedades funcionales, nutricionales o estructurales (Ozdal *et al*, 2013). El potencial antioxidante del guisante parece potenciarse durante la aplicación de calor debido a las interacciones fenol-proteína (Tsai & She, 2006), mientras que la adición de proteínas de leche disminuye la biodisponibilidad de los polifenoles del té (Lorenz *et al*, 2007). Los cambios conformacionales sufridos por las proteínas, derivados del procesado por calor y presión aplicados sobre anacardo, pistacho y castaña, podrían disminuir las interacciones de éstas con ciertos compuestos fenólicos de los frutos, favoreciendo así su extractabilidad (Ozdal *et al*¸ 2013).

De manera general, puede concluirse que los tratamientos de cocción 60 min, autoclave 121^{o}C 30 min y autoclave 138^{o}C 30 min, no afectan negativamente a la actividad antioxidante de los frutos secos analizados, e incluso incrementa significativamente la capacidad de absorción de radicales libres (ORAC) de castaña al ser ésta sometida a AU138^{o}C. En otros frutos secos y legumbres se ha observado cierta tendencia a incrementarse su capacidad antioxidante como consecuencia del procesado (Ranilla *et al*, 2010; Rodríguez-Bencomo *et al,* 2015). La formación de nuevos productos derivados de la reacción de Maillard, entre azucares reductores y grupos amino libres de proteínas, pueden ser responsables parciales de ese aumento en la capacidad antioxidante (Amarowicz, 2009).

En los últimos años se ha incrementado el interés en nuevos usos de harinas procedentes de ciertas semillas y frutos por su valor organoléptico y nutricional (Joshi *et al*, 2015). Ciertas harinas de leguminosas o de frutos como la castaña, se han planteado como alternativas a la de trigo (Demirkesen *et al*, 2010; Foschia *et al*, 2017). En este trabajo, hemos considerado interesante identificar las propiedades tecno-funcionales de las harinas de los frutos secos tratados, para determinar su posible uso como ingredientes en matrices alimentarias. Se seleccionaron los tratamientos de cocción 60 min y el autoclave a las condiciones más extremas, a 138^{o}C 30 min. Debido probablemente a cambios conformacionales de las proteínas y a la interacción de éstas con otros componentes, ciertas propiedades como la de retención y absorción de agua se incrementaron significativamente por la aplicación de calor, tanto de cocción como autoclave, en los tres frutos secos analizados. La mayor exposición de cadenas polares de los polipéptidos donde se puedan unir moléculas de agua con motivo del tratamiento explicaría parte de este incremento. La propiedad de retención de aceite experimenta la misma tendencia con los tratamientos excepto en castaña, en la que no se ve

incrementada con respecto a la harina sin procesar. Conviene destacar que la castaña posee ciertas cualidades considerablemente diferentes a otros frutos secos, como el contenido de agua, proteína, almidón o grasa (De Vasconcelos *et al*, 2010; Cruz *et al*, 2013).

En todos los casos, el tratamiento térmico y de presión afectó a las propiedades emulsionante y espumante, corroborando resultados anteriores (Alobo *et al*, 2009; Aguilera *et al*, 2011; Ling *et al*, 2016). La solubilidad de las proteínas y su conformación y estructura tienen una importante implicación en la capacidad emulsionante y espumante, y el tratamiento aplicado estaría influyendo sobre estos factores. Las harinas de anacardo y pistacho tratados mostraron la misma capacidad de gelificación que las harinas control, resultado no observado en castaña. Junto con el contenido de lípidos y carbohidratos, el nivel de globulinas parece estar relacionado con esta capacidad, siendo más del 50% del contenido de proteína en los tres frutos (Collada *et al*, 1986; Shokraii & Esen, 1988; Sathe & Sze-Tao, 1997).

Tomando los datos en conjunto, se puede concluir que las harinas de pistacho, anacardo y castaña con probada inmunoreactividad reducida conservan la mayor parte del contenido en compuestos fenólicos y poder antioxidante. Además, estas harinas desengrasadas mantienen algunas de las propiedades tecno-funcionales analizadas, lo que permitiría su uso para determinadas aplicaciones industriales. Sería relevante realizar un análisis sensorial y organoléptico de las harinas tratadas (con menor capacidad inmunoreactiva) de anacardo, pistacho y castaña para completar el estudio.

DETECCIÓN DE ALÉRGENOS DE FRUTOS SECOS MEDIANTE PCR EN TIEMPO REAL

Para proteger la salud de los consumidores alérgicos, el reglamento nº1169/2011 del Parlamento Europeo exige el correcto etiquetado de los ingredientes potencialmente alergénicos en todos los productos comercializados. Para cumplir dicha normativa, es necesario que agencias reguladoras e industrias alimentarias dispongan de métodos de detección sensibles, específicos y precisos para detectar ingredientes alergénicos que puedan estar presentes, intencionada o no, en los alimentos (De la Cruz *et al*, 2017). Los métodos basados en la detección de ADN han demostrado mayor adecuación que los métodos basados en proteínas para el análisis de alimentos muy procesados, dada la mayor estabilidad del ADN frente a determinado procesado (Iniesto *et al*, 2013; Prieto *et al*, 2014). El segundo objetivo principal de este trabajo es desarrollar métodos de detección de ADN basados en la técnica de PCR en tiempo real (RT-PCR) como estrategia de detección eficiente, sensible y específica de trazas de nuez (*Juglans regia)*, pistacho (*Pistacia vera*) y anacardo (*Anacardium occidentale*).

En este trabajo, se ha profundizado en el uso de secuencias codificantes de proteínas alergénicas como secuencias diana. Son generalmente genes de una o pocas copias, que proporcionan resultados específicos, en ocasiones comprometiendo la sensibilidad que se puede alcanzar empleando secuencias presentes en alto número de copias en el genoma, como las secuencias ITS en el genoma nuclear o secuencias del genoma de plastos y mitocondrias (Prado *et al*, 2012; López-Calleja *et al*, 2013; De la Cruz *et al*, 2013). Se han diseñado parejas de cebadores específicos de secuencias de genes codificantes de alérgenos de nuez (Jug r 1, Jug r 3 y Jug r 4), pistacho (Pis v 1, Pis v 2, Pis v 3 y Pis v 5) y anacardo (Ana o 1, Ana o 2 y Ana o 3).

En los tres trabajos que conforman este bloque, los cebadores y sondas se han diseñado específicamente analizando los alineamientos múltiples realizados con las secuencias obtenidas a partir de distintas variedades o cultivares de cada una de las especies (nuez, pistacho y anacardo). La zona seleccionada para el diseño de los cebadores/sonda está conservada en todas las variedades, minimizando así la aparición de falsos negativos (Costa *et al*, 2013a). Para diseñar los cebadores y sonda que garanticen un sistema específico, se ha tenido en cuenta también la variabilidad interespecífica. Para ello, se han analizado alineamientos múltiples de secuencias de nucleótidos que incluyen las obtenidas experimentalmente y las homólogas obtenidas de las bases de datos, pertenecientes a especies que se utilizan en industria alimentaria.

Independientemente del análisis *in silico* realizado, se ha evaluado empíricamente la especificidad de los cebadores en PCR en tiempo real (Bustin *et al*, 2009; Costa *et al*, 2014). En ocasiones se ha observado que pocas discrepancias en secuencias homólogas de distintas especies no son suficientes como para evitar la unión de los cebadores diseñados y se obtiene amplificación inespecífica no deseada. En los trabajos descritos se han incluido las secuencias de los cebadores y sondas diseñados, tal y como recomiendan las guías para la publicación de experimentos de PCR en tiempo real (Minimum Information for Publication of Quantitative Real-Time PCR Experiments, MIQE) (Bustin *et al,* 2009; 2010).

En nuez se han utilizado tres secuencias diana de forma paralela, determinando finalmente que la secuencia parcial del gen que codifica para una nsLTP, Jug r 3, arrojaba mejores resultados de sensibilidad. En pistacho y anacardo se ha realizado un cribado inicial de todos los cebadores Pis v y Ana o, seleccionándose Pis v 1 y Ana o 1 para realizar los ensayos completos. La determinación del límite de detección (LOD), de cuantificación (LOQ) y el análisis del método en muestras procesadas se ha realizado únicamente con esas secuencias diana. Otros trabajos de investigación encaminados a detectar frutos secos por RT-PCR han seleccionado secuencias codificantes de alérgenos, como Jug r 2 y Jug r 3 en nuez (Brežná *et al*, 2006; Costa *et al*, 2013b;),

Ana o 3 en el caso de anacardo (Brzezinski, 2006; Piknová & Kuchta, 2007; Ehlert *et al*, 2008), Pru du 1, 3, 5 o 6 en almendra (Costa *et al,* 2012; 2013b; Prieto *et al,* 2014;) o Cor a 1 y Cor a 8 de avellana (D'Andrea *et al,* 2011; Iniesto *et al*, 2013). Los LOD prácticos descritos en estos trabajos van desde 50 hasta 1 ppm del fruto en cuestión en distintas matrices. Este trabajo es el primero que plantea la detección de una secuencia parcial de un gen codificante de proteínas alergénicas de pistacho. Otros autores, por el contrario, han empleado secuencias multicopia como diana de detección (fundamentalmente ITS) para frutos de cáscara, incluyendo nuez y nuez pecana, anacardo y pistacho (Brežná *et al*, 2008; López-Calleja *et al*, 2014; López-Calleja *et al*, 2015b), obteniéndose LOD muy bajos, de incluso 0.1 ppm.

Tabla 4. Resumen de los resultados de sensibilidad obtenidos en los trabajos descritos para nuez, pistacho y anacardo. Se indica la química empleada en cada caso.*Porcentaje de réplicas con amplificación positiva a la concentración de harina de nuez, pistacho o anacardo que corresponda. N.D., no determinado.

Especie (secuencia diana)	Química	$LOD_{absoluto}$ (pg)	$LOD_{práctico}$ (ppm)*			LOQ ppm
			<75%	75-95%	95%	
Nuez (Jug r 3)	SYBR Green	2.5	N.D.	100	N.D.	500
Pistacho (Pis v 1)	SYBR Green	12.5	N.D.	N.D.	100	100
	Sonda-LNA	N.D.	N.D.	1	10	10
Anacardo (Ana o 1)	Sonda-LNA	2.5	5	N.D.	10	10
	Sonda-TaqMan	2.5	5	N.D.	10	10

En la tabla 4 se resumen los datos obtenidos en los tres trabajos descritos, respecto a la sensibilidad (LOD y LOQ) y las químicas de detección empleadas. Entre las químicas disponibles, se han utilizado las basadas en agentes intercalantes (SYBR Green) y en sondas de hidrólisis (LNA, TaqMan). El bajo coste y la facilidad de uso son las principales ventajas del SYBR Green, que se utilizó para el desarrollo de los métodos de detección de nuez y pistacho, obteniéndose LOD prácticos de 100 ppm en ambos casos y LOD absolutos de 2.5 y 12.5 pg de ADN respectivamente (con Jug r 3 y Pis v 1). Sin embargo, esta química de detección mostró ciertas limitaciones que condicionaron la especificidad y la sensibilidad del método en pistacho, fundamentalmente al discriminar entre trigo espelta y pistacho cuando este último estaba por debajo de 100 ppm en las mezclas binarias. Por ello, en pistacho se planteó el uso de sondas de hidrólisis fluorescentes, como una estrategia efectiva para minimizar y eliminar problemas de especificidad y mejorar la sensibilidad del método. Se han empleado sondas de hidrólisis LNA a partir de la librería de sondas UPL (*Universal Probe Library*, Roche), que pese a su pequeño tamaño, presentan la temperatura adecuada de hibridación para poder llevar a cabo la PCR (Echwald, Andreasen & Mourizen, 2016). Empleando este tipo de sondas, se incrementó el LOD práctico y LOQ en el sistema de detección de pistacho hasta 10 ppm mejorando los resultados obtenidos con SYBR Green. En este caso y en el trabajo descrito para detectar anacardo, se incrementa el nivel de confianza con respecto al trabajo de nuez, con amplificación positiva en al menos un 95% de las réplicas (Bustin *et al,* 2009). Fue posible detectar 1 mg de pistacho/kg de mezcla (1 ppm) en el

75% de las réplicas. En anacardo, se utilizaron estas sondas LNA, además de sonda TaqMan clásica. Se obtuvieron los mismos datos de LOD y LOQ que en pistacho, 10 ppm, en este caso detectando el mismo amplicón con sondas LNA y TaqMan. Fue posible detectar e incluso cuantificar hasta 5 mg/kg de anacardo en las mezclas binarias, aunque en menos del 75% de las réplicas para ambos sistemas (Tabla 4). En el trabajo de anacardo, se ha descrito la detección de ADN de almendra (con un valor Ct > 38) al emplear la sonda TaqMan, pero no con la sondas LNA. Esto parece indicar que, pese al análisis *in silico* realizado, debe existir un gen homólogo en almendra que presenta un número limitado de nucleótidos discrepantes con la secuencia diana. Estas discrepancias se encontrarían en la zona donde están localizadas las dos sondas (*ver Figura 1 del artículo 6*). La sonda TaqMan (de 24 nt) podría unirse a la secuencia semejante de almendra, pero las sondas tipo LNA confieren mayor especificidad, ya que no se unen a secuencias con las que difieran en un único nucleótido, lo que podría explicar este resultado. Otros trabajos han comparado la sensibilidad obtenida con las sondas TaqMan respecto a otras sondas para detectar dianas de interés. En la detección del patógeno *Campylobacter*, las sondas LNA fueron más sensibles que las TaqMan e incluso que las MGB o Scorpion (Josefsen *et al*, 2009). Algunos autores han mostrado previamente que las sondas TaqMan discriminan peor que otras sondas, como las MGB, diferencias de pocos nucleótidos (< 5) (Yao *et al*, 2006).

Influencia del procesado sobre la detección de frutos secos por RT-PCR

Dado que la mayoría de los alimentos sufre algún tipo de procesado durante su elaboración, se ha considerado necesario analizar la influencia de determinados tratamientos sobre el ADN de estos frutos y su detección mediante RT-PCR como paso previo al empleo de los sistemas de detección de secuencias de ADN para analizar presencia de nuez, pistacho y anacardo en productos alimenticios complejos y procesados. En los trabajos aquí descritos, se ha analizado el efecto de las altas presiones hidrostáticas (HHP) sobre la detección de nuez, de la cocción sobre pistacho y anacardo y de las distintas condiciones de autoclave sobre la detección de los tres frutos (121^{o}C/1.18 atm y 138^{o}C/2.56 atm, 15 y 30 minutos). El tamaño del amplicón ha sido en todos los casos, inferior a 100 pb lo que minimiza el efecto de la degradación del ADN como consecuencia del tratamiento aplicado sobre la amplificación de las secuencias diana (Hird *et al,* 2006).

En nuez se han realizado curvas estándar de ADN a partir de muestras tratadas con cuatro condiciones de HHP y de autoclave. Con los cebadores para Jug r 3, fue posible detectar nuez tratada por HHP sin comprometer la sensibilidad, con correcta eficiencia de reacción. Un resultado similar se había conseguido previamente en avellana cruda, en la que este tratamiento

a las mismas condiciones que las descritas, no afectó al a la detección de las secuencias diana (Iniesto *et al*, 2013). En el caso de la almendra, los tratamientos HHP afectaron a la integridad del DNA observándose que estaba fragmentado y probablemente su concentración esté sobreestimada (Prieto *et al*, 2014b). El efecto del tratamiento de HHP sobre la inmunoreactividad ha sido estudiado de distintos alimentos (Johnson *et al,* 2010; Husband *et al,* 2011). En el caso concreto de la nuez, la aplicación de las mismas condiciones de HHP que las descritas en este trabajo no supuso una reducción significativa de la capacidad inmunoreactiva del extracto proteico (Cabanillas *et al,* 2014)

En los trabajos de pistacho y anacardo se cambió el diseño experimental, analizándose curvas de mezclas binarias de harinas tratadas y considerando así el mismo efecto matriz que en la muestra control, con la que se ha establecido el LOD (Villa *et al*, 2018). Como ocurría con el procesado HHP en nuez, la cocción, tanto 30 como 60 min, no afectó considerablemente a la detección de pistacho y anacardo. López-Calleja y colaboradores (2014; 2015) obtuvieron resultados semejantes mediante PCR en tiempo real en muestras de pistacho, anacardo y nuez de Macadamia tostados a 160^{o}C durante 13 minutos, empleando ITS 1 como secuencia diana. Independientemente de la química empleada y del fruto seco analizado, se ha observado que el tratamiento de autoclave afecta a la amplificación de las secuencias diana. Este efecto es gradualmente mayor a medida que aumenta la temperatura, presión y tiempo aplicado. La eficiencia de la reacción obtenida a partir de curvas de nuez, pistacho y anacardo tratados con autoclave 121^{o}C 15 min, fue muy similar al control. Sin embargo, la sensibilidad se redujo respecto al control, al menos un orden de magnitud (10X). En el resto de tratamientos de autoclave, las especies objeto de estudio han mostrado diferente comportamiento y susceptibilidad al tratamiento. El análisis de las curvas de calibrado de ADN o harinas procedentes de frutos tratados con autoclave (121^{o}C 30') de nuez y pistacho respectivamente, permite discernir una disminución de la eficiencia de la reacción y se dificulta la cuantificación. En el caso de anacardo, sin embargo, se detecta y cuantifica este fruto tratado (121^{o}C 30 min) en un rango lineal de 100000 a 1000 ppm. A 138^{o}C/2.56 atm, la detección de nuez y pistacho se dificulta y la cuantificación no es posible. En anacardo, por el contrario, ha sido posible detectar el fruto a una concentración de 1000 ppm a partir de muestras tratadas a 138^{o}C 15 minutos. El pequeño tamaño del amplicón para Ana o 1 podría explicar también la capacidad de detectar y cuantificar ADN degradado por los tratamientos de presión aplicados (Costa *et al,* 2013b).

El ADN es una molécula más estable que las proteínas ante determinados procesados, y por ello, los métodos de detección basados en ADN se convierten en una estrategia poderosa para la detección de alérgenos en alimentos. Sin embargo, se ha descrito que la aplicación de

altas temperaturas y presión durante períodos considerables de tiempo, pueden afectar a la integridad del ADN y por tanto al resultado de la PCR, como el tratamiento de autoclave (Iniesto *et al,* 2013; Prieto *et al*, 2014). La influencia de determinados procesados sobre la detección de secuencias de ADN también se ha considerado un factor a analizar importante en alimentos que contienen OMG (Gryson *et al,* 2010). Como ya se ha mencionado previamente, algunas investigaciones han analizado el efecto sobre la integridad, cantidad, calidad y detección del ADN de componentes alergénicos, obtenido a partir de muestras tratadas (Iniesto *et al,* 2013; Prieto *et al,* 2014). También se ha planteado el análisis de muestras tratadas en mezclas binarias con harinas de otros ingredientes como trigo, de forma similar a lo aquí presentado en los trabajos para detectar pistacho y anacardo (López-Calleja *et al,* 2014; 2015). Otros autores han fabricado alimentos complejos en los que incluyen el ingrediente alergénico (*spiked)* para analizar el efecto de la matriz y del procesado térmico sobre la detección y cuantificación de alérgenos, como salchichas, galletas o pan (Hupfer *et al,* 2007; Platteau *et al,* 2011; Villa *et al,* 2018). Costa y colaboradores (2013b) fabricaron un bizcocho con concentraciones conocidas de nuez en la masa, para analizar el efecto del horneado sobre la detección de este fruto seco. Sus datos revelaron el mismo LOD y LOQ en muestras tratadas y sin tratar. Platteau *et al* (2011), por su parte, señalaron que el horneado afectaba negativamente a la sensibilidad del ensayo de RT-PCR con la diana Cor a 8 de avellana, resultado que también habían obtenido Scaravelli y colaboradores (2009) al procesar cacahuete mediante escaldado o tostado. Los trabajos aquí descritos incluyen una amplia batería de tratamientos térmicos y de presión aplicados sobre nuez, pistacho o anacardo, concluyendo que el efecto que estos tratamientos ejercen sobre la detección de las secuencias diana es variable en función de las condiciones de procesado.

La extracción y purificación de ADN a partir de alimentos es un factor determinante en el desarrollo de un método de PCR. En muestras complejas como son los alimentos es habitual la co-extracción de compuestos que pueden inhibir la reacción de PCR. La presencia de inhibidores de la reacción de PCR puede dar lugar a resultados falsos negativos, limitando la aplicabilidad del método desarrollado. Los protocolos de extracción descritos en los trabajos han permitido extraer ADN de buena calidad a partir de varios alimentos complejos como barritas de cereales, chocolate, galletas, masas y salsas. Sin embargo, la extracción de ADN a partir de ciertos alimentos con base de chocolate, se optimizó empleando un kit específico para este tipo de muestras (*NucleoSpin Food Kit*), con algunas modificaciones (Costa *et al,* 2015). Dicho protocolo se ha empleado para las muestras de alimentos analizadas con las dianas de pistacho y anacardo. Se incluyó un control positivo de amplificación (PAC) para confirmar que el

ADN extraído presenta la calidad y pureza adecuadas para la amplificación, para lo que se emplearon cebadores específicos del rDNA 18S de eucariotas.

Se han comparado los resultados de la detección de ADN por RT-PCR con la detección de proteínas específicas mediante ELISA para los tres frutos secos. En nuez, se analizaron las dianas Jug r 1, 3 y 4 y química SYBR Green, mientras que en pistacho y anacardo se empleó únicamente la diana, Pis v 1 y Ana o 1 respectivamente, con sondas LNA en ambos casos y TaqMan para anacardo. En todos los casos se incluyeron un mínimo de dos productos comerciales cuya etiqueta declaraba presencia del fruto a detectar, con resultados positivos tanto por PCR como ELISA. Los kits ELISA empleados presentaban reactividad cruzada con algún otro fruto seco, mientras que los métodos de detección desarrollados basados en RT-PCR han resultado más específicos, reduciéndose los falsos positivos y proporcionando un resultado más preciso. El procesado al que han sido sometidos algunos de los alimentos analizados han podido provocar degradación o solubilidad ineficiente de las proteínas diana del kit ELISA, y en consecuencia, se han obtenido falsos negativos mediante esta técnica, como el alimento "galletas con chocolate" en el trabajo presentado para detectar anacardo. Este problema también se ha podido solventar con los métodos de detección de ADN desarrollados. Resulta interesante destacar que se ha detectado la presencia de nuez en algunos alimentos que no etiquetaban trazas de ningún fruto seco, resultado corroborado por ELISA, lo que indicaría un etiquetado incorrecto que podría representar un riesgo importante para el consumidor.

Se puede concluir que los sistemas desarrollados de PCR en tiempo real permiten detectar trazas de nuez, pistacho y anacardo de un modo eficiente, específico y sensible. Los tratamientos térmicos de cocción no influyen considerablemente en la detección de Pis v 1 y Ana o 1, del mismo modo que el tratamiento no térmico de HHP sobre frutos de nuez no afecta a la amplificación de Jug r 3. Se demuestra que los métodos de detección de secuencias codificantes de alérgenos de nuez, pistacho y anacardo son aplicables en alimentos y matrices complejas, proporcionando datos sensibles y específicos.

CONCLUSIONES

De los resultados obtenidos en esta Tesis Doctoral se extraen las siguientes conclusiones:

1. La capacidad inmunoreactiva *in vitro* no se reduce en pistacho y anacardo procesados por cocción durante 30 y 60 minutos, aunque sí disminuye la capacidad de unir IgE en la superficie de células efectoras, determinada mediante pruebas cutáneas y ensayos de liberación de mediadores de la respuesta alérgica

2. El tratamiento térmico combinado con presión, de autoclave a 138^{o}C/2.56 atm durante 30 min, provoca una disminución drástica del reconocimiento de proteínas alergénicas de pistacho y anacardo por anticuerpos IgE. La capacidad para unir IgE en células efectoras e inducir liberación de mediadores también se reduce considerablemente respecto al control

3. Aunque algunos alérgenos de estos frutos secos presentan resistencia a la digestión enzimática, se ha demostrado que la hidrólisis con proteasas reduce la capacidad inmunoreactiva, especialmente de pistacho. La digestión enzimática aplicada sobre muestras procesadas con calor y presión disminuye considerablemente la capacidad alergénica *in vitro* de anacardo y pistacho. Estos tratamientos constituyen una herramienta efectiva para obtener pistacho y anacardo con capacidad alergénica muy reducida

4. El tratamiento de cocción disminuye el contenido de compuestos fenólicos de anacardo y pistacho, pero no afecta en castaña. Por el contrario, el tratamiento de autoclave lo mantiene, e incluso lo incrementa, con el aumento de la temperatura, la presión y el tiempo de tratamiento. El autoclave no disminuye el poder antioxidante en ninguno de los tres frutos secos analizados. Las harinas de frutos autoclavados mantienen algunas de sus propiedades tecno-funcionales, lo que las hace muy adecuadas para su uso en la industria alimentaria

5. Se han desarrollado métodos de detección de ADN de nuez, pistacho y anacardo por PCR en tiempo real. Para el diseño de los cebadores y sondas se han usado secuencias que codifican alérgenos. Para ello, en cada especie, se han seleccionado regiones conservadas intraespecíficamente y variables entre especies. Como sistema de

detección se han usado dos químicas, SYBR Green y sondas de hidrólisis (LNA y TaqMan)

6. Los métodos desarrollados permiten la detección, eficiente, sensible y específica de estos frutos secos, en mezclas binarias con harina de trigo espelta, alcanzando un LOD de 100 ppm para Jug r 3 de nuez, 10 ppm para Pis v 1 de pistacho y 10 ppm para Ana o 1 de anacardo

7. El procesado de pistacho y anacardo con cocción (30 y 60 minutos) no afecta a la detección de las secuencias diana. El tratamiento con altas presiones hidrostáticas (HHP) hasta 600 MPa tampoco ejerce un impacto negativo en la amplificación. Sin embargo, el tratamiento de autoclave dificulta la detección debido a su efecto sobre la integridad del ADN. A pesar de ello, los métodos de PCR en tiempo real desarrollados permiten detectar y cuantificar nuez y pistacho en harinas autoclavadas a 121^{o}C 15 minutos y anacardo en muestras autoclavadas a 138^{o}C 15 minutos

8. Se han validado estos métodos en alimentos comerciales procesados, obteniéndose resultados más precisos que con ensayos basados en la detección de proteínas (ELISA). La PCR en tiempo real resulta ser un método muy eficaz para asegurar el cumplimiento de la normativa vigente y proteger la salud de los consumidores alérgicos, así como detectar posibles alérgenos ocultos

CONCLUSIONS

From the results obtained in this Doctoral Thesis, the following conclusions have been drawn:

1. *In vitro* IgE-binding capacity is not reduced in boiled (30 and 60 minutes) pistachio and cashew nuts, although the ability to bind IgE on the surface of effector cells decreases, as determined by skin tests and mediator release assays

2. Combination of heat treatment with pressure, especially autoclave at 138^{o}C / 2.56 atm for 30 min, provoke a significant decrease in the recognition of pistachio and cashew allergen proteins by IgE antibodies. The ability to cross-link IgE on effector cells and mediator release induction is also diminished

3. Although some allergens of these tree nuts show resistance to enzymatic digestion, it has been probed that protease hydrolysis reduces IgE-binding capacity, especially of pistachio. The enzymatic digestion applied on samples processed with heat and pressure considerably reduces *in vitro* immunoreactivity of cashew and pistachio. These treatments are an effective tool to obtain pistachio and cashew with highly reduced allergenic capacity

4. Boiling processing reduces phenolic compounds content of cashew and pistachio, but does not affect chestnut. On the contrary, autoclaved samples show similar and even higher values than control, in parallel with the increase of temperature, pressure and time of treatment. Autoclave processing does not diminish the antioxidant capacity in any of the three analysed nuts. Autoclaved flours maintain some of their techno-functional properties, which makes them suitable for the use in the food industry

5. Real time PCR detection methods have been developed for walnut, pistachio and cashew DNA. For the primers and probes design, allergens coding sequences have been used. For each species, intraspecifically conserved regions but variable among different species have been selected. As a detection system, two chemistries, SYBR Green and hydrolysis probes (LNA and TaqMan) have been used

6. The real-time PCR methods allow the efficient, sensitive and specific detection of these nuts in binary mixtures made with *spelta* wheat flour, reaching an LOD of 100

ppm for Jug 3 from walnut, 10 ppm for Pis v 1 from pistachio and 10 ppm for Ana o 1 from cashew

7. Boiling processing of pistachio and cashew (30 and 60 minutes) does not affect the detection of the target sequences. The HHP treatment, up to 600 MPa, does not exert a negative impact on the amplification either. However, autoclave treatment makes detection difficult due to its effect on DNA integrity. In spite of this, the real time PCR methods allow the detection and quantification of walnut and pistachio in autoclaved flours at 121^{o}C for 15 minutes and cashew in autoclaved samples at 138^{o}C for 15 minutes

8. Applicability of these detection methods has been validated in processed commercial foods, obtaining more accurate results than those obtained by protein-based assays (ELISA). Real time PCR shows to be a very effective method to ensure compliance of current regulations in order to protect health of allergic consumers and to detect possible hidden allergens

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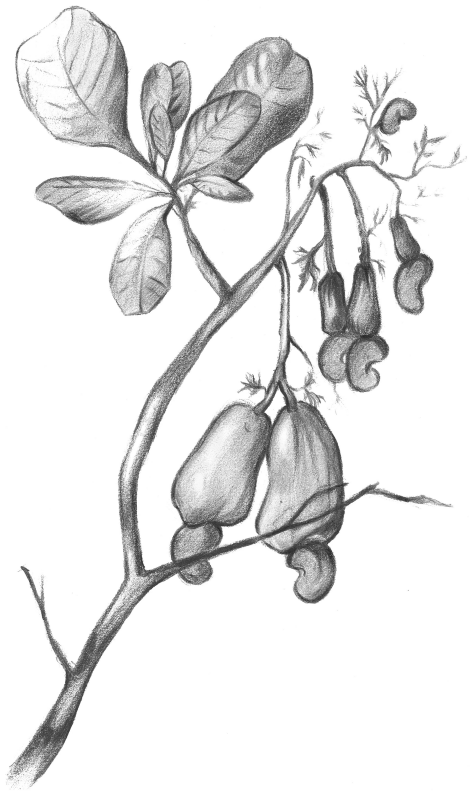

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